Fusion and cell entry by oncogenic sheep retroviruses

by

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To my parents,

For your unconditional support

I love you from the bottom of my heart
ABSTRACT

Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumour virus (ENTV) are two highly related oncogenic retroviruses that induce a contagious cancer in sheep and goats respectively. Both JSRV and ENTV use hyaluronidase-2 (Hyal2) as a cell entry receptor, yet JSRV induces lung tumours and ENTV causes tumours in the nasal epithelium. Unlike most acutely transforming retroviruses, the genomes of JSRV and ENTV do not contain an oncogene derived from the host cell; instead, the viral envelope protein (Env) functions as an active oncogene in addition of mediating cell entry. JSRV and ENTV Env are first synthesized as precursors that are cleaved by a cellular protease into two functional subunits: the surface (SU) subunit that contains the receptor binding domain and the transmembrane (TM) subunit that mediates membrane fusion. While most of previous studies have focused on the oncogenic properties of these proteins, little was known about how they mediate membrane fusion and viral entry. The goal of my Ph.D study was to investigate the mechanisms of fusion and cell entry by JSRV and ENTV Env as well as their regulations. We found that, although most retroviruses are believed to use a pH-independent pathway for entry, JSRV requires an acidic pH for entry and fusion and that its fusogenicity was negatively regulated by its cytoplasmic tail. Unexpectedly, ENTV Env requires an unusually low pH (<4.5) for fusion activation. While an irreversible inhibitor, Bafilomycin A1, which prevents acidification in the endosomes and lysosomes inhibited entry of JSRV and ENTV, ENTV Env-mediated infection was considerably enhanced in the presence of lysosomotropic agents or leupeptin, suggesting that JSRV and ENTV likely fuse in distinct cellular compartments. Importantly, we found that SU also modulates the fusion activity of JSRV and ENTV Env, despite that TM dictates the
differential pH requirements between JSRV and ENTV. The possible involvement of SU-receptor interaction in the fusion activation was further demonstrated by the fact that soluble receptor provided in trans can induce fusion of JSRV Env at low pH in normally fusion-inefficient cells, and that the soluble receptor induces SU shedding and modulates the TM conformational rearrangement induced by low pH. Taken together, our data indicate that JSRV/ENTV Env-mediated fusion is a controlled and multistep process, by which both receptor binding and low pH are required. The unique mechanism of JSRV/ENTV fusion activation and regulation might be important for their oncogenesis.
RÉSUMÉ

Le virus JSRV (Jaagsiekte sheep retrovirus) et ENTV (enzootic nasal tumor virus) sont deux rétrovirus oncogéniques apparentés qui induisent un cancer contagieux chez le mouton et la chèvre respectivement. Le récepteur utilisé lors de l’entrée de JSRV et d’ENTV dans la cellule cible est la protéine cellulaire hyaluronidase-2 (Hyal2). Cependant, alors qu’ils reconnaissent le même récepteur à la surface de la cellule, JSRV cause la formation de tumeurs au poumon tandis qu’ENTV induit l’apparition de tumeurs nasales. À l’opposé de la plupart des rétrovirus oncogéniques causant rapidement la formation de tumeurs, les génomes du JSRV et d’ENTV ne contiennent pas d’oncogène dérivé de la cellule hôte. Étonnamment, la protéine virale d’envelope (Env) joue un rôle d’oncogène actif en plus d’assurer ses fonctions durant l’entrée virale. Les Envs du JSRV et d’ENTV sont d’abord synthétisées sous forme de précurseurs qui seront éventuellement clivés dans l’appareil de golgi par une protéase cellulaire en ses deux sous-unités fonctionnelles : la sous-unité de surface (SU), qui contient le domaine de liaison au récepteur, et la sous-unité transmembranaire (TM) qui possède l’activité fusogénique. Alors que la plupart des études sur le JSRV et l’ENTV se concentrent sur les propriétés oncogéniques d’Env, les mécanismes par lesquels Env accomplit l’entrée virale et provoque la fusion de la membrane virale et la membrane cellulaire demeurent inconnus. Le but de ce projet de doctorat était d’étudier les mécanismes d’activation de la fusion ainsi que de mieux comprendre leur régulation. Alors qu’en principe la majorité des rétrovirus entrent dans la cellule via la fusion à la surface de la cellule à pH neutre, notre étude démontre que JSRV requiert un pH acide pour l’entrée et la fusion virale. De plus, l’activité fusogénique d’Env est régulée négativement par sa queue cytoplasmique. Étonnamment, l’Env d’ENTV
nécessite un pH anormalement acide (<4.5) pour l’activation de la fusion. En concordance avec le besoin d’un pH acide, la présence d’un inhibiteur irréversible qui empêche l’acidification des endosomes et des lysosomes inhibe l’infection par le JSRV et l’ENTV. Cependant, l’infection par l’ENTV est considérablement augmentée par des inhibiteurs réversibles de l’acidification des vésicules intracellulaires ainsi que par un inhibiteur de protéases (leupeptin), suggérant que JSRV et ENTV fusionnent probablement dans des compartiments intracellulaires distincts. Notre étude a également révélé que SU est responsable de la faible activité fusogénique d’ENTV et TM dicte le pH requis pour l’activation de la fusion. La participation de l’interaction Hyal2/SU dans le processus de fusion a été confirmée en utilisant le récepteur soluble qui cause l’induction de la fusion de cellules normalement réfractives, la dissociation de SU de son complexe avec TM ainsi qu’un changement conformationnel de TM. En somme, nos données indiquent que la fusion induite par l’Env de JSRV est un processus complexe impliquant l’interaction avec le récepteur en plus d’un pH acide et la régulation de ce mécanisme est possiblement importante à la pathogénèse de ces virus.
ACKNOWLEDGEMENTS

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I would like to thank my committee members, Dr. Chen Liang and Dr. Éric Cohen, for their assistance and advices. In addition, I would like to express my appreciation to all the researchers in the Lyman Duff I had scientific interactions with, especially Dr. Martin Olivier, Dr Sylvie Fournier and Dr Silvia Vidal. You were not directly involved in my supervision but you surely contributed to making me the scientist I am today.

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Preface

This thesis was written in accordance with McGill University’s “Guidelines for Thesis Preparation”. The candidate has chosen to present in a “Manuscript-based thesis” format following these recommendations:

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Presented in this thesis:


Other manuscripts not included in this thesis:


CONTRIBUTIONS OF AUTHORS

Chapter 2:
Fusogenicity of Jaagsiekte sheep retrovirus envelope protein is dependent on low pH and is enhanced by cytoplasmic tail truncations. Côté M, Zheng YM, Albritton L, Liu SL.
Chapter 2 is adapted from a published manuscript in the Journal of Virology and all data presented are derived from experiments performed by the candidate. Yi-Min Zheng generated the truncation mutants and Dr. Lorraine M. Albritton helped in the preparation of the manuscript. Dr. Shan-Lu Liu and the candidate designed the experiments and wrote the manuscript.

Chapter 3:
Enzootic nasal tumor virus requires a very acidic pH for fusion activation and infection. Côté M, Kurchaski TJ, Liu SL.
Chapter 3 is adapted from a published manuscript in the Journal of Virology and all data presented are derived from experiments performed by the candidate. Thomas J. Kucharski helped in the generation of the JSRV and ENTV Env chimeras. Dr. Shan-Lu Liu and the candidate designed the experiments and wrote the manuscript.

Chapter 4:
Chapter 4 is adapted from a submitted manuscript to the Journal of Virology and all data presented are derived from experiments performed by the candidate except for those involving the production and the purification of the soluble receptor which were done by Yi-Min Zheng. Dr. Shan-Lu Liu and the candidate designed the experiments and wrote the manuscript.
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<th>Description</th>
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<tbody>
<tr>
<td>6-HB</td>
<td>Six-helix bundle</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ASLV</td>
<td>Avian sarcoma and leukemia virus</td>
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<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>BafA1</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie and adenovirus receptor</td>
</tr>
<tr>
<td>CAT-1</td>
<td>Cationic amino acid transporter-1</td>
</tr>
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<td>CCP</td>
<td>Clathrin-coated pits</td>
</tr>
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<td>CCR5</td>
<td>Chemokine (C-C motif) receptor 5</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CMTMR</td>
<td>5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CT</td>
<td>Cytoplasmic tail</td>
</tr>
<tr>
<td>CVB</td>
<td>Coxsackie virus B</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine (CXC motif) receptor 4</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating factor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DC-SIGN</td>
<td>dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ds</td>
<td>double-strand(ed)</td>
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<tr>
<td>DSP</td>
<td>dithiobis[succinimidyl propionate]</td>
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<tr>
<td>DV</td>
<td>dengue virus</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EEA1</td>
<td>early endosome antigen 1</td>
</tr>
<tr>
<td>EIAV</td>
<td>equine infectious anemia virus</td>
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<tr>
<td>enJSRV</td>
<td>endogenous JSRV</td>
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<tr>
<td>ENTV</td>
<td>enzootic nasal tumor virus</td>
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<tr>
<td>Env</td>
<td>envelope</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FV</td>
<td>foamy virus</td>
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<tr>
<td>GALV</td>
<td>Gibbon ape leukemia virus</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GP</td>
<td>glycoprotein</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<td>GTP</td>
<td>guanosine-5′-triphosphate</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<td>HBZ</td>
<td>HTLV-1 b-ZIP</td>
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<td>HCV</td>
<td>hepatitis C virus</td>
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<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid</td>
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<td>HERV</td>
<td>human endogenous retroviruses</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HMW</td>
<td>high molecular weight</td>
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<tr>
<td>HN</td>
<td>hemagglutinin-neuraminidase</td>
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<tr>
<td>HR</td>
<td>heptad repeat</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>HTLV</td>
<td>human T-cells leukemia virus</td>
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<tr>
<td>Hyal2</td>
<td>hyaluronidase-2</td>
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<tr>
<td>ICAM-3</td>
<td>intercellular adhesion molecule 3</td>
</tr>
<tr>
<td>JSRV</td>
<td>Jaagsiekte sheep retrovirus</td>
</tr>
<tr>
<td>LMCV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>L-SIGN</td>
<td>liver/lymph node-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
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<td>MLV</td>
<td>murine leukemia virus</td>
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<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<td>MoMLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>M-PMV</td>
<td>Mason-Pfiser monkey virus</td>
</tr>
<tr>
<td>MSD</td>
<td>membrane spanning domain</td>
</tr>
<tr>
<td>OPA</td>
<td>ovine pulmonary adenocarcinoma</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology domain</td>
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<tr>
<td>PI</td>
<td>propidium iodine</td>
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<tr>
<td>PI(3)K</td>
<td>phosphoinositide-3- kinase</td>
</tr>
<tr>
<td>RBD</td>
<td>receptor binding domain</td>
</tr>
<tr>
<td>RDR</td>
<td>RD114/mammalian type D retrovirus receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Ron</td>
<td>récepteur d’origine nantais</td>
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</table>
SARS  severe acute respiratory syndrome
SDS  sodium dodecyl sulfate
Ser  serine
SFV  Semliki forest virus
SIV  simian immunodeficiency virus
SNAP  synaptosomal-associated protein
SNAREs  SNAP receptors
ss  single-strand(ed)
SU  surface subunit
SV40  simian virus 40
TBEV  tick-borne encephalitis virus
Thr  threonine
TK  thymidine kinase
TM  transmembrane subunit
VSV  vesicular stomatitis virus
CHAPTER 1

INTRODUCTION
1.1 Viral entry

Viruses represent a large group of infectious agents that varies in structure, genetic composition, cell entry mechanism, and replication strategy. While an extensive review of these differences is beyond the scope of this introduction, which mainly focuses on viral entry, a general understanding of viral replication is important because it determines to a certain extent how the progeny virions infect new cells.

1.1.1 Diversity in viral entry

Viruses are obligate intracellular microorganisms that require the cell and its machinery to replicate. Viruses vary greatly in size, shape and mode of replication. Outside the cell, they are found as a protein complex containing the genetic material of the virus. The protein shell, called capsid, can be made of one or more viral proteins that are encoded by the viral genome. Assembly of these proteins leads to diverse shapes, ranging from helical and icosahedral symmetry or bullet-shape morphology to a more heterogenous and long filamentous structure. The viral genome, composed of either RNA or DNA, is packaged inside the capsid, protecting itself from the extracellular environment. Some of them, called enveloped viruses, are surrounded by a lipid membrane derived from the host cell which contains the viral glycoproteins critical for viral entry [1]. This is an important distinction that dictates how the virus exits the infected cell and how it enters another cell. However, the internal structure of enveloped viruses is similar to their non-enveloped counterparts. Table 1.1 illustrates the diversity in the strategies employed by viruses. Note that the site of entry and replication are representatives of the particular examples, and that it does not reflect the situation for all viruses of the same family.
### Table 1.1: Diversity in viral entry and replication

<table>
<thead>
<tr>
<th>Baltimore classification</th>
<th>Family</th>
<th>Env</th>
<th>Site of replication</th>
<th>Virus example</th>
<th>pH dependence</th>
<th>Site of entry</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. dsDNA</td>
<td>Poxviridae</td>
<td>Y</td>
<td>cytoplasm</td>
<td>vaccinia virus</td>
<td>Y</td>
<td>Macropinocytosis/Endosome</td>
<td>[2]</td>
</tr>
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<td></td>
<td>Asfarviridae</td>
<td>Y</td>
<td>cytoplasm/nucleus</td>
<td>african swine fever virus</td>
<td>Y</td>
<td>Endosome</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>Herpesviridae</td>
<td>Y</td>
<td>nucleus</td>
<td>herpes simplex virus</td>
<td>N</td>
<td>Plasma membrane</td>
<td>[4]</td>
</tr>
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<td></td>
<td>Papovaviridae</td>
<td>N</td>
<td>nucleus</td>
<td>SV40</td>
<td>N</td>
<td>Caveolae/ER</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>Adenoviridae</td>
<td>N</td>
<td>nucleus</td>
<td>adenovirus5</td>
<td>Y</td>
<td>CCP/Endosome</td>
<td>[6]</td>
</tr>
<tr>
<td>II. ssDNA</td>
<td>Parvoviridae</td>
<td>N</td>
<td>nucleus</td>
<td>parvovirus</td>
<td>Y</td>
<td>Endosome</td>
<td>[7]</td>
</tr>
<tr>
<td>III. dsRNA</td>
<td>Birnaviridae</td>
<td>N</td>
<td>cytoplasm</td>
<td>infectious pancreatic</td>
<td>N</td>
<td>Endosome</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>Reoviridae</td>
<td>N</td>
<td>cytoplasm</td>
<td>reovirus</td>
<td>Y</td>
<td>Endosome</td>
<td>[9, 10]</td>
</tr>
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<td>IV. ssRNA(+)</td>
<td>Coronaviridae</td>
<td>Y</td>
<td>cytoplasm</td>
<td>severe acute respiratory</td>
<td>Y</td>
<td>Endosome</td>
<td>[11, 12]</td>
</tr>
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<td></td>
<td>Caliciviridae</td>
<td>N</td>
<td>cytoplasm</td>
<td>feline calicivirus</td>
<td>Y</td>
<td>Endosome</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>Astroviridae</td>
<td>N</td>
<td>cytoplasm</td>
<td>serotype-1 astrovirus</td>
<td>Y</td>
<td>CCP/Endosome</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>Togaviridae</td>
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<td>cytoplasm</td>
<td>semliki forest virus</td>
<td>Y</td>
<td>CCP/ Early endosome</td>
<td>[15, 16]</td>
</tr>
<tr>
<td></td>
<td>Flaviviridae</td>
<td>Y</td>
<td>cytoplasm</td>
<td>dengue virus</td>
<td>Y</td>
<td>CCP/ Late endosome</td>
<td>[17]</td>
</tr>
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<td></td>
<td>Picornaviridae</td>
<td>N</td>
<td>cytoplasm</td>
<td>coxsackie B virus</td>
<td>N</td>
<td>Caveolae</td>
<td>[18]</td>
</tr>
<tr>
<td>V. ssRNA(-)</td>
<td>Orthomyxovirida</td>
<td>Y</td>
<td>nucleus</td>
<td>influenza virus</td>
<td>Y</td>
<td>CCP/Late endosome</td>
<td>[19, 20]</td>
</tr>
<tr>
<td></td>
<td>Rhabdoviridae</td>
<td>Y</td>
<td>cytoplasm</td>
<td>vesicular stomatitis virus</td>
<td>Y</td>
<td>CCP/Endosome</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>Paramyxovirida</td>
<td>Y</td>
<td>cytoplasm</td>
<td>newcastle disease virus</td>
<td>N</td>
<td>Plasma membrane/ Caveolae</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>Arenaviridae</td>
<td>Y</td>
<td>cytoplasm</td>
<td>lassa virus</td>
<td>Y</td>
<td>Late endosome/lysosomes</td>
<td>[23]</td>
</tr>
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<td></td>
<td>Filoviridae</td>
<td>Y</td>
<td>cytoplasm</td>
<td>ebola virus</td>
<td>Y</td>
<td>CCP/caveolae</td>
<td>[24, 25]</td>
</tr>
<tr>
<td></td>
<td>Bunyaviridae</td>
<td>Y</td>
<td>cytoplasm</td>
<td>hanta virus</td>
<td>Y</td>
<td>CCP</td>
<td>[26]</td>
</tr>
<tr>
<td>VI. ssRNA(+)</td>
<td>Retroviridae</td>
<td>Y</td>
<td>Nucleus</td>
<td>human immunodeficiency virus-1</td>
<td>Y/N*</td>
<td>Plasma membrane/</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Hepadnavirida</td>
<td>Y</td>
<td>Nucleus</td>
<td>hepatitis B virus</td>
<td>N</td>
<td>Endosome</td>
<td>[28]</td>
</tr>
</tbody>
</table>

Env: Envelope   Ref: References   Y: Yes   N: No   CCP: Clathrin Coated Pit  
* Long assumed to fuse at the surface at neutral pH, a growing number of retroviruses are found to use low pH for entry. HIV entry is pH-independent and fusion occurs at the surface of the cell or possibly in the endosomes [27].
1.1.2 *Viral life cycle*

While each family of viruses exhibits individual characteristics, the overall viral life cycle can be divided into: adsorption and penetration of the host cell, replication of the genome and viral protein synthesis and, finally, assembly and release of the progeny virions [1].

The first step in viral entry involves the attachment of the virions to the host cell. Attachment is achieved by the binding of viral surface proteins to host receptors or attachment factors. The interaction with the receptor is specific and an important determinant of the tropism of the virus [29]. There is a wide range of attachment factors and receptors used by viruses, and this will be discussed in more details in section 1.1.3. Typically, binding to the receptor generates structural changes of the viral surface proteins, and attachment is followed by penetration [30]. The orthomyxoviruses are particular, because they possess a surface viral protein, the neuraminidase (NA), which exhibits exoglucohydrolase activity [31]. These viruses bind to sialic acid-containing proteins at the surface of the target cell, however if they do not proceed to fusion; the virus can release itself from the cell using the neuraminidase activity of NA at the surface of the virion. NA hydrolyses terminal sialic acid residues and, as a result, the virus leaves and infects another cell. [32]. Although this mechanism can be useful in escaping from unsuccessful entry, it is mostly used during viral release from the infected cells [33].

Following receptor binding at the surface of the host cell, the virus can either penetrate the cell directly by fusion of the viral membrane with that of the cell, or be internalized by endocytosis that is followed by penetration via intracellular vesicles [29]. Fusion at the plasma membrane can only be executed by enveloped viruses, while endocytosis is required for non-enveloped viruses or enveloped viruses which require extra
cues for fusion activation, such as an acidic pH or host pH-dependent proteases [30]. Endocytosis of viral particles is an important entry pathway for many viruses, and will be discussed in depth in section 1.1.4. The endocytosed virions, once in the appropriate environment, will initiate penetration of the host cell by either membrane fusion for enveloped viruses, or by pore formation or membrane lysis for non-enveloped viruses [29].

Penetration is followed by uncoating which allows genome expression and replication. Viral genome replication strategies vary greatly among viruses. Most DNA viruses, ss or ds, replicate in the nucleus where they can use the host cell DNA polymerase to produce new progeny viral genomes, and the host RNA polymerases for the generation of messenger RNAs for the production of the viral proteins [1]. Most RNA viruses, ss and ds, replicate in the cytoplasm [34]. However, unlike double-stranded and positive-sense single-stranded RNA viruses, the negative-sense RNA viruses need to first transcribe their genomes into a positive-sense RNA molecule using their viral RNA-dependent RNA polymerases [1]. The host polymerase then uses this template for production of the viral proteins. The retroviruses use a different mode of replication where the RNA genome is reverse-transcribed into DNA by the viral enzyme, reverse transcriptase, and this DNA provirus is integrated in the host genome with the help of another viral enzyme, the integrase [35]. Retroviruses will be discussed in more details in section 1.5.1. The hepadnaviruses also display a distinct mode of replication. In this case the DNA genome is reverse-transcribed by a viral polymerase into an RNA intermediate, which will be use for viral protein production and DNA genome replication [36].

The subcellular location and mechanisms of viral assembly vary among viral families. However, in general, the virions are built so that they can be triggered for the infection of a new cell without the need of additional energy [29]. This concept is
particularly important for enveloped viruses that require the viral glycoprotein in a metastable state for fusion. Most of the viruses require the proteolytic cleavage of the fusion protein, or a companion glycoprotein prior to budding [37].

The newly assembled viruses exit the cell through multiple means: budding, apoptosis, lysis, and exocytosis. The latter three mechanisms are used primarily by non-enveloped viruses. Budding provides enveloped viruses with their membrane containing the viral glycoproteins (except for poxviruses [38]). Budding occurs mostly at the surface of the host cell, but can also take place in intracellular compartments or organelles, followed by exocytosis [39-41]. In many cases, the newly released virions, especially in the case of retroviruses, need to undergo a maturation step by the cleavage of viral proteins by the viral protease [42].

1.1.3 Entry receptors and co-factors

As mentioned above, the initiation of the viral life cycle requires the recognition of one or more cellular receptors and/or co-factors. The interaction with receptors is required for both enveloped and non-enveloped viruses, and the receptor used by these viruses is an important determinant of the tropism of the virus. The types of receptors used by viruses are extremely diverse, which can range from lipids and carbohydrates moieties to membrane proteins with different topology [9, 29, 43, 44]. In addition, while most viruses use a single specific receptor, some viruses require multiple receptors or co-factors for entry. In many cases, viral entry is assisted by the triggering of signalling cascades in the cell that allow the virus to traffic in the host cell [29]. This is generally achieved by direct interaction with the host receptor and/or clustering of signalling molecules [29].
1.1.3.1 Attachment factors

Attachment factors are cellular molecules that help the virus to bind to the host cell. The interaction between the virus and the attachment factor is generally not specific, and weak [45]. However, the interaction with multiple attachment factors could have an additive effect that enhances the avidity, resulting in an irreversible binding of the virus to the host cell [46]. One important characteristic of attachment factors is that they do not trigger the mechanism of viral entry. For instance, HIV binds initially to the cell through association with attachment factors, including mannose binding C-type lectin receptor family members; the dendritic cell-specific intercellular adhesion molecule (ICAM)-3 grabbing non-integrin (DC-SIGN), liver and lymph node-specific ICAM-3 grabbing non-integrin (L-SIGN) [47], but these interactions do not cause initiation of viral fusion or any conformational change in the viral glycoprotein, leading to fusion. Instead, the fusion process is triggered only upon interaction with the cellular receptor and co-receptors of the virus, CD4 and CXCR4 or CCR5 [48, 49].

1.1.3.2 Receptor diversity

Membrane proteins and carbohydrates. Transmembrane proteins are widely used as receptors for viral entry. Since these are cellular membrane-associated proteins, they are subjected to glycosylation. While some viruses bind to the carbohydrate sidechains of the glycoprotein, others bind specifically to regions of the amino acid backbone of the protein. Viruses exploit all types of membrane proteins, which include: single transmembrane proteins, multipassed transmembrane proteins and glycosylphosphatidylinositol (GPI)-anchored proteins [44]. For viruses requiring endocytosis for entry and penetration, the receptor they use is often critical for targeting the virus to specific internalization route.
Thus, the use of a transmembrane protein instead of a GPI-anchored protein can be critical for virus entry, which can lead to either a productive or a non-productive infection.

Several viruses use specific carbohydrate-containing molecules as receptors such as sialic-acid containing proteins, glycosaminoglycans or glycolipids [50-52]. For instance, herpes viruses, adeno-associated viruses (AAV), dengue virus (DV), and others have been shown to bind heparan sulfate [53-56]. Interestingly, for some of them, the binding is sensitive to the degree of sulfatation [56]. In addition, modification by host specific sulfotransferases can modulate the efficiency of binding of the virus [57]. However, most often, carbohydrates on host proteins or lipids mainly serve as attachment factors, and then interactions with the viral proteins do not normally trigger the initiation of viral penetration [9].

For enveloped viruses, carbohydrates are not only present on the host receptor but they are also on the viral protein. The viral proteins found at the surface of enveloped viruses are frequently heavily glycosylated [35]. Whereas glycosylation may play a role in protein folding, intracellular trafficking, stability, and immune evasion, glycosylation of the viral protein is mostly critical for viral binding to the host cell, which modulates the degree of interaction with the receptor [58]. The most frequent glycosylation is the N-glycosylation, with the addition of oligosaccharide groups to the nitrogen of an asparagine side chain found in a Asn-X-Ser/Thr motif [59]. The addition of sugar groups occurs early in protein synthesis, and is followed by a complex rearrangement and trimming in the endoplasmic reticulum (ER) and golgi apparatus [59]. As a result, the viral glycoprotein possesses varying oligosaccharide structures that can be important for its function [60]. Glycosylation near or at the receptor binding site has been reported to alter viral binding to their cognate receptors for several viruses, such as influenza virus and HIV [32, 61, 62].
Interestingly, some viruses bind to host lectins, and the viral glycoprotein contains the carbohydrate ligand. For example, human cytomegalovirus (CMV), HIV-1, DV, hepatitis C virus (HCV), and Ebola virus, bind to host lectins DC-SIGN and L-SIGN [47, 63-67]. As such, the type of N-glycosylation added to the viral protein, such as high mannose or complex carbohydrates can affect the binding to those lectins and modify the tropism of the virus, as has been shown for Ebola virus [68]. This feature is quite important, since it implies that the type of glycosylation, which is dependent on the virus-producer cells, can enlarge the viral tropism and could have important consequences on the pathogenicity of the virus.

*Lipids.* The envelope protein of vesicular stomatitis virus (VSV) is a choice of predilection for pseudotyping because of its extremely wide tropism. The reason for this wide host range lies in the cell entry receptor it uses. While still controversial, it is believed that the entry receptor for VSV is phosphatidylserine. Indeed, it has been shown that binding of the virus to target cells could be inhibited by the addition of membrane extracts [69]. These extracts maintained their inhibitory activity even after heating or protease and neuraminidase treatment, but were shown to be unable to block viral binding if the extracts were treated with phospholipase C. Furthermore, the addition of purified phosphatidylserine was able to specifically inhibit VSV infection [69]. However, one study suggested that phosphatidylserine is not involved in the recognition of the target cell and that the real cell entry receptor has yet to be determined [70]. In any case, it is generally believed that phosphatidylserine is important for viral glycoprotein interaction with the membrane [43] and that it is at least involved in a post-binding step of the entry process.
1.1.4 *Viral entry pathways*

Viruses have evolved to use a multitude of entry pathways to enter the host cell, and some of the cellular endocytic processes were in fact discovered from the study of viral entry. In some cases, for enveloped viruses in particular, entry may occur at the cell surface through fusion of the viral and plasma membrane. For others, receptor binding leads to the internalization of the virion-receptor complex, and access to the cytoplasm occurs via membrane fusion or pore formation in intracellular vesicles or organelles. The entry pathways used by viruses are diverse and the study of which has led to a better understanding of cellular processes as well as to the discovery of new pathways or intracellular compartments, such as the caveosome (reviewed in [29]).

1.1.4.1 *Entry via the cell surface*

Upon binding to their cognate receptor, some enveloped viruses can enter directly the host cell by membrane fusion at the plasma membrane. This mean of entry is used by the majority of paramyxoviruses, herpesviruses and most retroviruses [4, 35, 71, 72]. While fusion at the surface may seem easy and simple, it involves multiple disadvantages. For instance, this mode of entry is not suitable for viruses that require low pH, proteases, or a particular redox state for fusion activation [46]. Therefore, it can only be used by viruses that are activated solely by receptor binding. In addition, a consequence of fusion at the plasma membrane is that viral glycoproteins still remain at the cell surface, which can be recognized by the immune system at an early stage of the infection. For non-enveloped viruses that induce membrane lysis for penetration, the destruction of the endosomal membrane is likely less damaging to the host cell than lysis of the plasma membrane. Probably one of the most important disadvantages of entry at the cell surface is the need to
navigate through the strong barrier of cortical actin underneath the surface. By contrast, viruses using the cellular endocytosis machinery can easily avoid this difficulty, and can be delivered closer to its site of replication, which is especially important for viruses that replicate in the nucleus [46].

While it was long assumed that HIV-1 required only receptor binding for cell entry, a recent study showed elegantly that HIV-1 does not enter the cell by the fusion of the viral membrane with the cell surface membrane but requires endocytosis, which was dynamin-dependent, with complete fusion occurring in the endosomes. This entry pathway was characterized by the necessity of receptor binding, not only for fusion activation but for receptor-mediated internalisation as well [27]. Therefore, the exact entry compartments of other viruses assumed to require only receptor binding should be re-examined.

1.1.4.2 Clathrin-mediated endocytosis

The entry pathway that is most widely used by enveloped or naked viruses is the clathrin-mediated, dynamin-dependent endocytosis. Uptake through this route generally leads to viral entry in early endosomes, and late endosomes. Most viruses that use this pathway require an acidic environment to initiate either fusion of the viral membrane with the vesicular membrane, or pore formation [29]. Therefore, the clathrin-mediated route is efficient because it enables viral delivery to an acidic milieu within minutes following endocytosis. Clathrin-mediated endocytosis is a constantly ongoing process in the cell, and while some viruses may internalize via already-assembled clathrin-coated pits, others were shown to be able to induce its formation [73, 74]. For instance, semliki forest virus (SFV), the first virus that was demonstrated to use clathrin-mediated endocytosis for entry, and influenza virus were both shown to be able to induce signalling events leading to formation
of clathrin-coated pits underneath the bound viral particles [15, 75]. Several kinases and signalling molecules can affect the internalization rate of clathrin-dependent endocytosis. Consequently, viral uptake through this route is often stimulated by the virus through different means such as receptor-mediated signalling.

Endocytosis via the clathrin-mediated pathway normally first delivers the virus to early endosomes and requires the small GTPase protein, Rab5 [29, 76]. These vesicles are characterized by the presence of the EEA1 marker and display an internal pH around 6.5 to 6.0 [29]. Therefore, early endosomes are often the site of penetration for viruses requiring a slightly acidic milieu, such as SFV (requires a pH around 6.2) [16]. For viruses exhibiting a lower pH threshold for penetration, internalized viral particles are brought to late endosomes, which contains the small GTPase protein, Rab7, and a pH range from 6.0 to 5.5 [29]. Thus, it is the site of fusion with cells used by several enveloped viruses such as influenza (optimum pH of around 5.0) [20, 77]. The progression of the endocytosis pathway leads to delivery to lysosomes. While lysosomes have been generally regarded as a dead end for viruses leading to viral degradation instead of productive infection, it is now clear that some viruses use this intracellular compartment for entry. Lassa virus has been recently shown to require an extremely low pH of around 4.0 for fusion suggesting that this virus may use lysosomes as a site of entry [23]. Moreover, lysosomes are enriched in proteases such as cathepsin L and B necessary for Ebola virus and SARS infection [24, 78, 79]. While late endosomes can also contain these proteases, the exact penetration site of these viruses is still unclear.
Another very important entry route used by viruses is the caveolar/raft pathway. The first virus identified to utilize this pathway for infection is the simian virus 40 (SV40) [80]. For internalization, SV40 binds to its receptor, major histocompatibility (MHC) class I molecules leading to the relocalisation of MHC-I in to caveolae domains. Unlike clathrin-mediated endocytosis that is often constitutive, the caveolae-mediated endocytosis is triggered exclusively [81]. Following its relocalisation into the caveolae, the virus induces intracellular signalling that involves local tyrosine phosphorylation and rearrangement of the cortical actin network resulting in recruitment of actin and dynamin-2 to the plasma membrane. The signalling cascade induced by mechanism results in a slow and selective internalization of the virus in a small vesicle routed to the caveosome [80]. Intriguingly, the MHC class I molecule is not internalized with the virus suggesting the presence of a secondary receptor in SV40 infection [82]. A caveosome is a neutral membrane-bound cytoplasmic organelle enriched in cholesterol and caveolin-1. This compartment is negative for markers of other intracellular vesicles involved in trafficking such as all Rab GTPases [80]. The dynamics of the caveosome is tightly linked to signalling events. For instance, under normal conditions, the caveosome is static with a poor efficiency at exchanging or recycling caveolin-1 molecules. However, upon SV40 signalling, the caveosome becomes more active. After trafficking of the internalized virus in a caveolae vesicle to the caveosome, the virus is released from the caveosome in transport vesicles that do not contain any caveolin. These vesicles are then transported to the smooth ER where uncoating of SV40 takes place [80].

While SV40 in the caveosome is sent to the smooth ER, the caveosome also communicates with the endosomal trafficking pathway. Endosomes have been shown to
contain a small amount of caveolin-1 and to associate to caveolin-1-enriched vesicles. The relationship between those two pathways could be critical for viruses that require low pH for penetration, yet use the caveolar/lipid raft for internalization. For instance, the avian sarcoma/leukosis virus (ASLV) uses a distinct endocytosis pathway for entry depending on the form of the receptor it interacts with [83]. The transmembrane version of the receptor leads to rapid endocytosis via the classical clathrin-mediated entry route, whereas a GPI-anchored form of the receptor mediates a slow entry via the caveolae, the latter being an internalization pathway similar to the one exploited by SV40. Importantly, regardless of the entry pathway taken, ASLV requires low pH to activate its fusion machinery [84, 85]. Consequently, the virus is to be trafficked to the acidic endosomes for penetration into the host cell, clearly suggesting a communication between the caveolar/lipid raft internalisation route and the endosomal trafficking pathway.

In addition of sorting viruses to their sites of uncoating, caveosomes can also be the site of penetration for some viruses. Echoviruses also exploit caveolae-mediated endocytosis for entry [86]. During infection, the virus first bind to $\alpha_2\beta_1$ integrins and is then internalized via calveolae [87]. Interestingly, echovirus 1 uptake is faster than that of SV40 and does not appear to require actin remodelling [88]. These differences therefore suggest heterogeneity in the caveolar/lipid raft entry pathway. Importantly, echovirus 1 is not sorted to other intracellular organelles or vesicles. Instead, this virus initiates penetration and release of its genome from the caveosome [88].
1.1.4.4 Non-clathrin, non-caveolea entry pathways

Viruses are not restricted to use a single pathway for entry. For instance, while the majority of influenza particles enter cells through clathrin-mediated endocytosis, some fractions utilize a clathrin-independent pathway for internalization [75]. This pathway is shared by the arenavirus lymphocytic choriomeningitis virus (LCMV) [89]. The molecules involved in the regulation of this pathway and how they are modulated are still not well understood. However, there is evidence that this process requires the presence of cholesterol and is not necessarily dynamin-dependent. Interestingly, SV40 is able to infect cells that lack caveolae, and this process is clathrin-independent [90]. Therefore, this pathway can traffic the internalized vesicles to both the endosomes and the caveosome. In conclusion, the non-clathrin, non-caveolea mediated entry pathway seems very heterogeneous, and can lead to more than one fate. However, the overall process, whether it uses dynamin or not or wherever the cargo is delivered, seems to be modulated by a small group of kinases suggesting that it is interconnected as a whole [91]. Further study of this pathway will possibly allow the identification of subsets of unique internalization routes.

1.1.5 Virus-induced signalling in the host cell

Viruses trigger signalling pathways during the entry process. This is true for viruses that fuse at the plasma membrane, or require internalization prior to penetration. The first viruses reported to use host signalling pathways for entry are adenoviruses that bind to their main receptor, the coxsackie and adenovirus receptor (CAR), and to their co-receptors, integrins [92]. It was shown that the interaction between the virus and integrins triggers the phosphatidylinositol 3-kinase (PI(3)K) pathway, leading to further activation of signalling cascades involving Rac and Cdc42. The activation of Rac and Cdc42 triggers actin
remodelling and endocytosis of the virus via clathrin-coated vesicles [6, 92-94]. The Ebola virus also activates the PI(3)K pathway to promote its internalisation, yet the endocytosis pathway it uses still unclear and may involve both the clathrin-mediated endocytosis and the caveolae [25, 95].

As briefly mentioned in previous sections, other internalization routes used by viruses, such as the raft/caveolar-mediated endocytosis, also require the activation of signalling cascades. Another virus that exploits the caveolar pathway is the coxsackie virus B (CVB). The co-receptor of CVB is a GPI-anchored protein; the decay accelerating factor (DAF) [96]. The binding of the virus with DAF causes the clustering of the receptor and the initiation of an intracellular signalling cascade involving the activation of the tyrosine kinase c-abl which, in turns, triggers Rac [18]. Activation of Rac leads to actin remodelling, enabling the virus to be transported from the apical surface to the tight-junctions of the cell where the virus can interact with CAR. This results in the activation of Fyn that phosphorylate caveolin-1 and internalization of the virions via the caveolar pathway [18]. Therefore, signalling cascades can be initiated by either direct binding to a receptor, exemplified by the interaction of adenoviral proteins with integrins, or by the clustering of proteins or lipids in lipid rafts, exemplified by the cross-linking of DAF induced by the binding of CVB.

1.2 **Membrane fusion and fusion proteins**

One critical step in the entry of enveloped viruses into the host cell is the fusion of its viral membrane with that of the host cell. This process is mediated by viral fusion proteins that overcome the energy barrier of membrane fusion by a conformational
rearrangement that involves the formation of bundles. Importantly, the study of virus fusion is critical for the understanding of other membrane fusion events. Indeed, membrane fusion is a general process involved in several critical biological functions, such as intracellular vesicles fusion, and cell-cell fusion. Interestingly, while at first glance these processes seem very different, they share similar features [30, 97].

1.2.1 Membrane fusion

Fusion is characterized by the union of the lipid bilayer of two membranes resulting in the mixing of their respective contents. For fusion to occur, several energy barriers have to be overcome. It is now generally believed that membrane fusion proceeds through a hemifusion intermediate (Fig.1.1) [98, 99]. The first step is to bring the two lipid bilayers in close proximity in order to generate a first intermediate, the fusion stalk, where the external phospholipid layers make a local contact. A challenging task is to overcome the strong repulsive force generated when the two membranes are in a distance below 2nm. This repulsion is caused by the strong binding of water to the lipid polar head groups, known as the hydration force [100]. Although bringing the two membranes closer is energy consuming, the formation of the fusion stalk has a low energy cost compared to the steps that follow [101]. In addition, stalk formation is favoured depending on the lipid composition of the membranes. For instance, the cone-shaped phosphadidylethanolamine (PE) is better than the inverted-cone-shaped phosphatidylcholine (PC) in inducing the fusion stalk [98]. The second step is the radial enlargement of this zone of contact, which leads to the merging of the two outer leaflets of the phospholipid bilayers. This intermediate is called hemifusion, characterized by partial lipid mixing without content mixing [102]. The expansion of the hemifusion diaphragm leads to the fusion of the outer
leaflets and formation of a small pore and small content mixing. This small pore can spontaneously close back, by a process called pore flickering. Finally, the last step is pore expansion which permits the mixing of bigger components such as a viral capsid. Theoretical analysis and calculations demonstrated that the enlargement of the fusion pore requires more energy than the formation of the hemifusion diaphragm [98, 103]. Generally, the high energy required for fusion between the two membranes is driven by fusion proteins.
Figure 1.1. Membrane fusion. A) The current model of fusion of two lipid bilayers involves a hemifusion intermediate. The two membranes are placed in close proximity and form an initial contact of fusion of the outer layers of the two respective bilayers termed “fusion stalk”. The expansion of the surface contact of the fused outer layers of phospholipids leads to hemifusion. Further expansion results in the opening of a fusion pore. The consequence of the enlargement of the fusion pore is the formation of a small pore. Whereas all the previous steps are thought to be reversible, further enlargement into a large pore of around 5nm in diameter or more is irreversible. Figure adapted from Judith M White & J David Castle, Nat. Struct. Mol. Biol. 2005 [104]. B) Energy diagram of the fusion between two membranes. Figure borrowed and modified from Stephen C. Harrison, Nat. Struct. Mol. Biol. 2008 [105].
1.2.2 Viral fusion proteins

Enveloped viruses enter cells by fusing their membrane with that of the host cell. This process is mediated by one or more viral glycoproteins at the surface of the virions. While divergent in sequences, structural studies revealed that the fusion proteins of unrelated viruses are conserved in structure (Reviewed in [30, 106]). According to their structure and their oligomeric state, three classes of fusion proteins have emerged. The characteristics of each class of fusion proteins with some examples are summarized in Table 1.2. Although there are clear distinctions between the three classes of viral fusogens, a common characteristic of all viral fusion proteins is the formation of a trimer of hairpins in the post-fusion conformation.

Table 1.2. The three classes of viral fusion proteins.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examples</td>
<td>Retrovirus Env, Orthomyxovirus HA, Paramyxovirus F, Filovirus GP, Arenavirus GP, Coronavirus S</td>
<td>Alphavirus E1, Flavivirus E</td>
<td>Rhabdovirus G, Herpes virus gB, Cytomegalovirus gB, Baculovirus gp64</td>
</tr>
<tr>
<td>Predominant secondary structure</td>
<td>α-helix</td>
<td>β-sheet</td>
<td>α-helix &amp; β-sheet</td>
</tr>
<tr>
<td>Fusion peptide</td>
<td>At or near N-terminus and buried at the intersubunit interface</td>
<td>Internal fusion loop buried at the dimer interface</td>
<td>Bipartite fusion domain made of exposed fusion loops</td>
</tr>
<tr>
<td>Proteolytic processing</td>
<td>Processing of fusion protein</td>
<td>Processing of companion protein</td>
<td>No cleavage</td>
</tr>
<tr>
<td>Post-fusion conformation</td>
<td>Trimer of hairpins with a central α-helical core</td>
<td>Trimer of hairpins made of β-sheet domains</td>
<td>Trimer of hairpins with a central α-helical core</td>
</tr>
<tr>
<td>Conformational change during fusion</td>
<td>Metastable trimer to stable trimer</td>
<td>Metastable dimer to stable trimer</td>
<td>Non-metastable trimer to trimer</td>
</tr>
<tr>
<td>References</td>
<td>[107]</td>
<td>[108-110]</td>
<td>[111-114]</td>
</tr>
</tbody>
</table>
1.2.2.1 Class I viral fusion proteins

The prototype and one of the most studied class I viral fusion proteins is influenza virus hemagglutinin (HA). Class I fusion proteins also includes retroviral envelope (Env) protein and paramyxovirus F protein [105]. All class I fusion proteins are composed mainly of α-helices, and are found at the surface of the virions as trimers in their pre-fusion conformation. They are initially synthesized as precursors that trimerize, and require cleavage by a cellular enzyme, furin or furin-like enzyme, during traffic to the plasma membrane surface [30]. In some cases, such as the F protein of the paramyxovirus Hendra virus, the cleavage is mediated by cathepsins shortly after recycling of the viral glycoprotein from the surface into intracellular vesicles [115]. In general, the cleavage generates two fragments that are often linked together by a disulfide bond [30]. For some viruses, one surface subunit that contains a receptor binding domain is responsible for attachment. It is generally believed that the surface subunit acts as a clamp, and inhibits the transmembrane subunit from refolding [30]. The transmembrane subunit contains a fusion peptide at or near the N-terminus and two heptad repeats, and possesses the fusogenic activity. The fusion peptide is a stretch of hydrophobic amino acids with an alpha-helical structure (discussed in more details in section 1.4.2). After cleavage of the precursor, the fusion protein is found in a metastable state. Upon specific triggers, the fusogenic potential of the fusion subunit is relieved and the protein refolds into the first intermediate, the pre-hairpin or rod-like structure, which leads to the insertion of the fusion peptide in the target (host) membrane (see figure 1.2). Following the first conformational change, the protein refolds into a hairpin structure where the N- and C-terminal heptad repeats interact. The interaction between the N- and C-terminal heptad repeats of each monomer of the trimer results in a trimer of hairpins or 6-helix bundle (6-HB) formed by a central core of N-
heptad repeats surrounded by the C-heptad repeats. The formation of the hairpins pulls the
two membranes in close proximity and ultimately leads to hemifusion and fusion of the
viral and cellular membranes. While there are striking structural similarities between the
members of the class I fusion proteins, the position of the 6-HB varies, and the triggers they
require for fusion activation are also very diverse [30].
**Figure 1.2. Fusion by class I fusion proteins.** (A) Influenza HA fusion activation requires conformational rearrangement of HA1 and HA2. In the pre-fusion state, the fusion peptide of HA2 is buried at the subunits interface. Upon a low pH trigger, HA1 is displaced resulting in the refolding of HA2 in a rod-like structure. The extension of HA2 leads to insertion of its fusion peptide into the outer leaflet of the lipid bilayer of the target membrane. A second conformational change involves the refolding of the extended alpha-helical core into a hairpin structure, whereby the C-terminal and the N-terminal heptad repeats interact. (B) Model of fusion-mediated by class I fusion proteins. In the pre-fusion conformation, the ectodomain of the fusion protein is made of a C-terminal central alpha-helical core. Following the formation of the rod-like structure, in response to a specific trigger, the protein refolds into a hairpin as explained in (A). The formation of the fusion pore possibly necessitates the coordinated action of more than one trimer of hairpins, which displays a central N-terminal alpha-helical core in the post-fusion conformation. *Figures (A) and (B) adapted from Stephen C Harrison Nat Struct Mol Biol 2008.* [105]
A) Pre-fusion
B. Low pH, dissociation of HA1
C. Formation of the rod-like structure, Insertion of the fusion peptide
D. Formation of the six-helix bundle, Pore formation
E. Interaction between the fusion peptide and the transmembrane region

B) A. Pre-fusion
B. Pre-hairpin or rod-like structure
C. Formation of the hairpin
D. Hemifusion
E. Six-helix bundle or post-fusion conformation
1.2.2.2 Class II viral fusion proteins

Unlike the class I fusion proteins, E1 and E are composed mainly of β-sheets, contain an internal fusion peptide, and lie low at the surface of the virions in a metastable state (see figure 1.3 and reviewed in [116]). The alphavirus E1 and flavivirus E proteins were proposed the founding members of the class II fusion proteins with the most characterized being the semliki forest virus (SFV) E1 protein and the flavivirus tick-borne encephalitis virus (TBEV) E protein [116]. The crystal structures of TBEV E, SFV E1 and Dengue virus (DV) E proteins reveal that the ectodomain can be divided into three globular domains [109, 117-119]. Domain I is at the N-terminus and forms a β-barrel that is connected by a flexible hinge region to the elongated domain II. Domain II contains the fusion loop bordered by two β-strands and four conserved disulfide bonds. Domain I is also connected to domain III via a small linker region. Domain III is a β-barrel, containing 3 disulfide bonds, and is connected to a stem region that anchors the fusion protein to the membrane via the transmembrane region [116]. During synthesis, the fusion protein normally associates with a viral chaperone protein, for examples p62 and prM of SFV E1 and TBEV E respectively, forming a heterodimer in the pre-fusion state [120, 121]. This interaction causes the fusion peptide to be buried at the dimer interface. Another difference from the members of the class I, is that the fusion proteins of the class II are not proteolytically cleaved. Instead, the chaperone protein is the one requiring proteolytic cleavage [122]. For instance, the p62 protein of alphaviruses is cleaved during transit to the cell surface in the trans-golgi network by the cellular protease furin generating the mature E2 [120, 123]. In the case of flaviviruses, which bud in the endoplasmic reticulum, the incorporated prM protein is cleaved by furin when the virus is trafficked to the surface for
exocytosis [124]. Flaviviruses are distinct from alphaviruses in that during a step called maturation, the fusogenic protein dissociates from its companion protein and forms an E-E homodimer that is fusion competent [125].

Upon activation, class II fusion proteins undergo dramatic conformational rearrangements. All class II fusion proteins known to date require low pH for fusion. For SFV, exposure to low pH causes the dissociation of E1 from its chaperone protein E2, leading to the exposure of the fusion loop contained in E1 [126]. This, in turn, induces the realignment of E1 and projection from the viral membrane to the target membrane by the insertion of the fusion loop. Interestingly, insertion of SFV E1 fusion domain is highly dependent on lipid composition, and cholesterol presence has been shown to be required for SFV fusion [127, 128]. Importantly, the horizontal to vertical reorientation of E1, triggers its homotrimerization with a central core composed of domain I and II [129]. Similar to class I fusion protein, the SFV E1 then refolds to form a trimer of hairpins by the folding back of domain III towards the central core. The formation of the hairpin brings the two membranes into close proximity, leading to the creation of a hemifusion diaphragm and full fusion [116].
Figure 1.3. Fusion mechanism of a prototype class II fusion protein. The regions of the protein colored in red, yellow and blue correspond to domain I, II and III respectively. In the pre-fusion conformation (A), Dengue virus E protein is found as a homodimer. Upon low pH, the protein dimer is believed to dissociate and to form an extended monomeric intermediate (B), leading to insertion of the fusion loop of domain II into the target membrane and trimerisation (C). Domain III then folds back against domain I, thereby forming the trimer of hairpins in the post-fusion conformation. Figure adapted from Stephen C. Harrison, Nat. Struct. Mol Biol. 2008. [105]
1.2.2.3 Class III viral fusion proteins

Recently, a new class of viral fusion proteins has been assigned for vesicular stomatitis virus (VSV) G, herpes simplex virus-1 (HSV-1) gB, Epstein-Barr virus (EBV) gB, and baculovirus gp64 proteins since they share similarities reminiscent to proteins of both class I and class II [111-114] (reviewed in [130]). Similar to class I fusion proteins, these proteins are found at the surface of virions as trimers and contain a central α-helical core. Yet, like class II fusion proteins, the fusion peptides of these proteins are internal and found at the tip of β-sheet folds. The fusion peptide is certainly one of the very distinct features of the members of the class III fusion proteins, since their fusion domains are bipartite, i.e. they are composed of two fusion loops.

Similar to class II fusion proteins, VSV-G does not required proteolytic processing for fusion activation. However, reminiscent to class I, it trimerizes in the ER, which allows its trafficking to the golgi apparatus and ultimately to the surface of the cell for its incorporation onto budding virions [131]. Interestingly, although it is present as a trimer, its fusion loops are not buried at the trimer interface; instead they are exposed and found outside the protein (see figure 1.4). The VSV-G ectodomain is divided into five domains. Domain I, also called the fusion module, contains the bipartite fusion peptide. Domain I is followed by domain II that exhibits a pleckstrin (PH)-like fold. PH domains serve as interaction platform with phospholipids or proteins [132], yet, whether or not domain II can bind cellular molecules important during the fusion process is still unknown. Domain II is linked to domain III, which forms the central α-helical core reminiscent of class I fusion proteins. Domain III is connected to domain IV that consists in a potentially flexible region. The structure of the latter domain is extremely varied among the different class III fusion
proteins. Finally, domain V is found at the C-terminus of the ectodomain and is link to the membrane spanning domain. Because the ectodomain of VSV-G used for crystallisation is a thermolysin-cleaved fragment, the structure of VSV-G does not contain domain V due to removal by proteolysis. However, spectroscopic analysis of this region revealed that it is mostly unstructured, yet contains some α-helical structures similar to the other class III fusion proteins [133].

In the pre-fusion state, the fusion loops, located at the tip of domain I, are oriented towards the viral membrane [112]. Upon triggering in an acidic environment, the protein undergoes two phases of massive conformational rearrangements, which include insertion of the fusion domain in the target membrane and fusion of the latter with the viral membrane. The first conformational change involves the structural modification of two linkers. The unstructured linker I, joining domain IV to domain III, becomes α-helical leading to a 90° rotation of domain IV toward the target membrane. The linker II that connects domain III to domain II and adopts initially an α-helical structure, becomes unstructured leading to the 90° rotation of domain III. The end result is the positioning of the fusion domain directly towards the target membrane [134]. The second phase of conformational change is modulated the rearrangement of an unstructured loop in domain II into an α-helix that folds back and interacts with the central core, thereby forming the overall hairpin structure [112]. This results in the reorientation of the fusion peptide toward the viral membrane, dragging the target membrane at the same time in close proximity to the viral membrane and ultimately leading to fusion [134]. One unique feature of rhabdovirus glycoproteins such as VSV-G, is that they do not exist in a metastable state, and the conformational rearrangement VSV-G undergoes at low pH is reversible [135].
**Figure 1.5. Fusion mechanism of class III fusion proteins.** (A) Crystal structures of the four actual members of class III fusion proteins. Domains I to V are shown in blue, green, yellow, orange and red respectively. Please note that domain V of VSV-G crystal structure is missing. The positions of the two linkers of VSV-G important in the conformational change are indicated by arrows. (B) In A, in the VSV-G pre-fusion conformation, the fusion domain (marked by an asterix) is displayed on the outside of the trimer. Upon low pH exposure, domain I flips over, leading to the formation of the extended intermediate, and thereby inserting the fusion domain in the target membrane. A second change in conformation leads to the formation of the hairpin and fusion of the viral membrane with that of the cell. *Figure A adapted from Backovic, M. and Jardetzky, T. S. Curr. Opin. Struct. Biol.* [130] *Figure (B) adapted from Stephen C. Harrison, Nat. Struct. Mol Biol.* 2008. [105]
1.2.3 Cellular fusion proteins

The study of viral fusion proteins provides important information for the understanding of other more complex membrane fusion events. The fusion of two membranes is critical in several biological functions, such as intracellular vesicles fusion and cell-cell fusion.

1.2.3.1 SNAREs, vesicular fusion

For the proper transport and trafficking of cellular proteins directed to the secretion pathway or for the transport of incoming endocytosed components, cells require a specialised machinery that allows fusion between intracellular vesicles. Conserved in eukaryotic cells, fusion of intracellular compartments is mediated by a large family of proteins called SNAP receptors (SNAREs) [136, 137]. SNAREs are small type II transmembrane proteins with the bulk of the protein exposed to the cytoplasm, a membrane spanning domain, and, for most of them, a small region facing the extracellular milieu or the lumen of the vesicle [138]. Their fusing elements are contained in the cytoplasm, within what is called the SNARE motif that contains two long heptad repeat regions joined by a linker termed the O layer. According to the O layer, SNAREs are classified into 4 different groups: Qa, Qb, Qc and R-SNAREs [139]. The Q-SNARES are usually found on target membranes, and generally termed t-SNAREs, while the R-SNAREs are normally found in the opposing membrane, which is the one of the transport vesicles, known as the v-SNAREs [138]. It has been shown that cognate t- and v-SNAREs are sufficient to induce fusion of liposomes and cells, without any additional input of energy [140, 141]. The driving force for the fusion of the vesicles is the conformational rearrangement and the complex formation of two cognate SNAREs, which release enough energy to overcome the
repulsive forces existing between the two membranes. In its pre-fusion state, the SNAREs are separated, and found unfolded [142]. However, when two cognate SNAREs assemble, a complex of a parallel four-helix bundle is formed by the interaction of the two heptad repeats found in each SNARE [143]. This conformation is stable, and some complexes can resist SDS treatment and heat [144]. These conformational and complex rearrangements pull the two membranes in close proximity, leading to fusion. Similar to viral fusion proteins, the conformational rearrangement releases enough energy to mediate fusion. Interestingly, the dissociation of the SNAREs complex for recycling requires energy, because of the high stability of the post-fusion state [145]. This stable post-fusion conformation is reminiscent of almost all fusion proteins, and cellular SNAREs share features with class I fusion proteins.

1.2.3.2 Oocyte and Sperm fusion, Osteoclasts and Myoblast fusion

While the first step in the life of an individual is the fusion between the oocyte and the spermatozoid, during development, other cell-cell fusion events are necessary; such as myoblast fusion for the formation and repair of the multinucleated myofibers [146]. The molecular mechanisms underlying these processes are still unclear but seem to require members of the large family of A disintegrin and metalloproteinase (ADAMs) proteins [147, 148]. All ADAMs contain at least four potential functional domains: prodomain, metalloprotease-like domain, disintegrin-like domain and cysteine-rich domain, and they play different roles and functions in proteolysis, adhesion, fusion and signalling [147]. A subset of ADAMs contains a potential fusion domain, and is likely involved in cell-cell fusion. One of these is ADAM 1 (also named fertilin α) is important in oocyte and sperm fusion during fertilization [149]. Another one is ADAM 12, which seems to play a role in
osteoclast and trophoblast fusion, and myogenesis [150, 151]. Interestingly, their fusion domains contain a potential fusion peptide, which is a stretch of 23 hydrophobic residues, embedded in a cysteine-rich region reminiscent of viral fusion proteins [152]. In addition, these fusion peptides can be modeled into either an alpha-helix or beta-sheet (see section fusion peptide 1.4.2) [153]. However, whether ADAM 1 and 12 possess membrane fusion activity remains to be determined.

1.2.3.3 Trophoblast fusion, formation of the syncytiotrophoblast of the placenta

During pregnancy, most mammals form a layer of multinucleated cells that is critical for healthy pregnancy. This layer separates maternal and fetal blood, and serves to regulate the transport of oxygen, nutrients, waste, and also produces hormones required for the continuation of the pregnancy [154]. In humans, it was hypothesised that ADAM 12 is involved in this process [155], but the most important candidate is the Env of endogenous human retroviruses HERV-W and HERV-FDR [156]. The HERV-W element became part of the primate genome around 25 million years ago. The Env gene of this retroviral sequence is functional, and has been named syncytin-1 [157]. Its receptor is RDR, the mammalian type D retrovirus receptor which is an amino acid transporter [158]. HERV-FDR is an older element that entered the genome 40 million years ago [159, 160]. Its Env protein, termed syncytin-2, is also functional and is expressed in the placenta [160]. These envelope proteins induce cell fusion in vitro [160], and syncytin-1 has been shown to play a direct role in trophoblast fusion [161]. Interestingly, similar endogenous retroviral sequences have also been shown to be involved in rodent syncytiotrophoblast formation, and possibly in that of sheep as well.
1.3 **Fusion Activation**

Viral fusion proteins are present at the surface of the virions in a native state or pre-fusion state, and they need specific triggers to release their full fusion potential. Typically, fusion proteins can be triggered by receptor interaction, referred to as pH-independent, or by low pH, termed pH-dependent. It is now clear that the mechanisms of fusion activation are more diverse than originally thought. For instance, ASLV requires both receptor binding and low pH, while Ebola virus necessitates cleavage by host pH-dependent proteases for entry [106].

1.3.1 *pH-independent*

The pH-independent fusion proteins are triggered by the receptor and/or co-receptor binding, and viral fusion normally occurs at the cell surface. For instance, most retroviruses require the interaction of a single receptor for entry. One example is Moloney murine leukemia virus (MoMLV) which has been extensively studied, and involves thiol reaction exchange during the fusion process. In some cases, fusion proteins, such as the HIV envelope protein, require the sequential binding of multiple receptors for fusion and entry. Moreover, other fusion proteins, such as the viral fusion proteins of paramyxoviruses, do not bind directly to receptors; instead their activation requires the interaction of another viral protein to the host receptor [106].

1.3.1.1 *MoMLV Env, requirement of a single receptor*

Like all retroviral Env, MoMLV Env is a class I fusion protein containing a surface (SU) and a transmembrane (TM) subunit, which remain linked by a disulfide bridge [162].
SU contains the receptor binding domain (RBD) and recognizes the cationic amino acid transporter-1 (CAT-1), and TM is responsible for the fusion [163]. Importantly, the SU subunit contains a CXXC motif following a proline-rich hinge region, domains that are highly conserved among retroviruses [162, 164]. The TM subunit contains a CX\(_6\)CC motif, in which two out of the three cysteines form an intramolecular disulfide bond with the remaining one linked to one of the CXXC cysteine of SU, leaving one cysteine free. It has been shown that the free cysteine is stabilised by the binding of Ca\(^{++}\)[162]. The CXXC motif has been found in protein disulfide isomerases, and is the basis of the isomerase activity of Mo-MLV Env [162, 165].

During MoMLV entry, the interaction between the receptor and the Env RBD located at the N-terminal region of SU causes a conformational change in the SU, which is transmitted to the proline-rich region and subsequently to the CXXC motif. The conformational change causes Ca\(^{++}\) release, which leaves a highly reactive free thiol of the unlinked cysteine, leading to isomerisation of the disulfide bond at the CXXC motif [166]. Consequently, the disulfide bond between SU and TM is disrupted, and a new intramolecular disulfide linkage is formed between the two cysteines of the CXXC motif. It has been shown that deletion of the RBD of SU results in a fusion-deficient Env protein. Interestingly, soluble RBD provided in \textit{trans} is sufficient to render the Env fusogenic, suggesting that a signal mediated by the RBD after receptor binding is necessary to induce the Env fusion activity [167, 168]. The rearrangement in disulfide bonds also leads to SU shedding, which liberate its restriction on TM for fusion activation, and leads to TM conformational change and ultimately fusion [169]. Recently, it was shown that cathepsin L potentiates MoMLV infection by cleaving the SU subunit [170]. It has been hypothesized that cleavage of SU increases the propensity of TM to refold.
1.3.1.2 *HIV gp120 and gp41, interaction with receptor and co-receptor*

The HIV glycoprotein is first synthesized as a precursor, gp160, which is cleaved in the golgi apparatus into the surface gp120 and transmembrane gp41 subunits. Unlike MoMLV, the two subunits composing HIV Env remain associated through non-covalent interaction. Indeed, there is no disulfide linkage between gp41 and gp120, and gp120 does not contain a CXXC motif, although many intramolecular disulfide bonds are present in both subunits [171]. The gp120 subunit, for example, contains 9 disulfide bonds [171]. Thus, HIV Env does not appear to exhibit an intrinsic isomerase activity as the MoMLV Env does. However, a role for cell surface protein reductases in HIV infection has been proposed [172].

HIV infection requires the presence of the receptor CD4 and one of these two co-receptors: CCR4 or CXCR5, depending on the tropism of the virus, and viral fusion is believed to occur at the surface of the host cell [173]. As discussed in a previous section, this view of surface fusion for HIV-1 has been recently challenged [27]. Nevertheless, the general fusion mechanism of HIV-1 Env is still valid. An interaction of HIV Env with its main receptor, CD4, induces conformational rearrangement in gp120, allowing binding to its co-receptor [174]. Indeed, primary receptor binding exposes the co-receptor binding site, or alters it in a way that allows binding. Interestingly, this conformational change is stable, and gp120 maintains its ability to bind the co-receptor long after the CD4 trigger. It is believed that it is co-receptor binding that allows the final conformational change in gp120, and releases gp41 fusion potential. Is it not entirely clear if this involves shedding of gp120 from the complex [175, 176]. Nonetheless, gp120’s conformational change after co-
receptor binding must lead to exposure of gp41 fusion peptide and ultimately to the formation of the six-helix bundle and fusion [177].

1.3.1.3 Paramyxoviruses, binding of a receptor by an independent viral protein

In the case of most paramyxoviruses and herpes viruses, two independent viral glycoproteins are involved in the fusion activation. One protein, often called the attachment protein, mediates binding to the receptor, and another possesses the fusogenic properties. For paramyxoviruses, the attachment protein, HN or G, are present as homotetramers at the surface of the virus [178]. Similar to all class I fusion proteins, the fusogenic glycoprotein, F, is processed into F1 and F2, which remain linked by a disulfide bond and found as a homotrimer at the surface of virions. F1, the transmembrane subunit, contains the fusion peptide and heptad repeats, and mediates fusion [178].

How paramyxovirus fusion is induced is still controversial. In the first model, the receptor binding glycoprotein does not interact, or interacts with low efficiency with the fusogenic proteins in the pre-fusion state. Upon binding to the host cell receptor, the attachment protein undergoes a conformational change, leading to its tight association to the F1/F2 complex. This interaction triggers the change in conformation of F1 which allows fusion peptide insertion, and subsequent 6-helix bundle formation [179]. In the second model, the attachment protein is associated with the F1/F2 complex, and inhibits its fusion activity. In this case, binding to the receptor causes dissociation of the attachment protein from the fusion complex. The liberation of the inhibitory effect of the attachment protein leads, in turn, to the activation of the F1/F2 complex [180]. While more evidence support the second model, by which receptor binding causes dissociation and activation of the fusion complex, contradictory data also support the first model. Therefore, the exact
mechanism, by which paramyxovirus fusion is activated, is still controversial. Given that there are some paramyxoviruses that use a protein receptor while others use carbohydrates for attachment; there is a possibility that the fusion activation mechanism varies between the different species of paramyxoviruses [181, 182]. In addition, some paramyxoviruses have been shown to induce virus-cell and cell-cell fusion at low pH [183]. In conclusion, many questions still remain regarding the paramyxovirus fusion mechanism.

1.3.1.4 Herpes virus glycoproteins

Fusion of herpes viruses involves several key players and studies have shown that four out of the twelve viral glycoproteins are involved in fusion. These include gD, gB, and the gH/gL heterodimer [184]. Fusion activation of herpesviruses resembles, to some extent, that of paramyxoviruses. The gD contains the receptor binding domain, and gB contains the bipartite fusion peptide characteristic of the new class III fusion proteins [185]. However, gB does not seem to be the sole player in membrane fusion since the gH/gL complex has been shown to participate in the fusion process. A recent study reported that, in the pre-fusion state, gD interacts with the other proteins to form complexes of gD/gB and gD/gH/gL respectively. After receptor binding, gD dissociates from its partners and a new complex of gB/gH/gL is generated [186]. In addition, another study showed that upon receptor binding, gD transmits a signal to gH/gL which induces hemifusion and gB causes full fusion [187]. However, the effect of receptor binding on gD and how gB and gH/gL coordinate membrane fusion are still unclear. Moreover, HSV infection of certain cell types requires a low pH trigger [188]. Whether the overall fusion mechanism is the same in those conditions is unknown.
1.3.2 **pH-dependent**

Viruses using a pH-dependent pathway for entry require receptor binding to the host cell surface, endocytosis, and vesicular trafficking in order to reach an intracellular acidic compartment for fusion. Several recent studies have highlighted the importance of histidine residues in the pH-dependent fusion activation (reviewed in [189]). Indeed, most pH-dependent viral fusion proteins fuse at an acidic pH around 6.0, consistent with the pKa of histidine, which is around 6 to 7, yet varies depending on the surrounding residues [190]. Thus, histidine residues can be pH sensors that are potentially very important in the low pH-induced conformational change. At neutral pH, histidine residues are mostly found uncharged with their nitrogen forming a hydrogen bond with positively charged residues. At pH 6.0 or below, histidines become double protonated, and therefore destabilize the original interaction through electrostatic repulsion. In addition, this newly acquired charge may promote new interactions, such as salt bridges with other residues. It is hypothesized that these new interactions are more stable, partly explaining the irreversibility of the process [190]. The potential involvement of histidine residues has been demonstrated for several pH-dependent viruses such as influenza virus, TBEV, VSV and others [191-193]. Mutations of these residues were shown to affect the pH threshold or the fusion activity. It is interesting to note that not only some critical histidines are conserved in HA of the different influenza strains, but the surrounding amino acids as well [190]. Therefore, the hypothesis of histidine residues playing a key role in low pH-induction of fusion is gaining more and more credibility.
1.3.2.1 Influenza hemagglutinin (HA), low pH trigger

Influenza HA is the prototype of class I fusion proteins, and its conformational change induced by low pH has been extensively studied. HA is found in a metastable state at the surface of the virions due to proteolytic cleavage of the HA precursor in the infected cells [194]. Cleavage of HA generates HA1 and HA2, which are linked by a disulfide bond. The surface subunit HA1 is responsible for attachment to sialic acid-containing proteins and for clamping the fusion activity of the transmembrane subunit, HA2 [195].

Binding of HA to the cell surface does not trigger any significant conformational changes in the glycoprotein, yet binding allows the endocytosis of the virus in order to reach an acidic intracellular environment in the host cell [196]. Instead, low pH causes protonation of histidine residues in HA1 and HA2, leading to the dissociation of the two subunits [197, 198]. The separation, although not complete, of the two subunits is thought to release the inhibition placed on HA2, resulting in HA2 conformational change and ultimately fusion [105, 198]. Thus, low pH is the sole trigger for HA fusion activity. As a result, pre-treatment of virions with a low pH buffer leads to an irreversible change in the conformation of HA2 and therefore to viral inactivation [199]. The inactivation of the virions by low pH in the absence of the target membrane is an important feature of typical pH-dependent viruses.

1.3.3 Requirement of both receptor binding and low pH

ASLV Env requires both receptor binding and low pH for fusion activation [200]. This is unprecedented since it was always thought that fusion activation necessitates receptor interaction at neutral pH or the acidic environment of intracellular vesicles, but not
both. While ASLV is the only virus confirmed to use such a fusion activation mechanism, there is a possibility that others may use a similar method of activation.

### 1.3.3.1 **ASLV uses a unique two-step mechanism for fusion**

ASLVs are simple retroviruses that belong to the alpharetrovirus genus and is divided into ten subgroups, A to J, depending on their receptor usage [201]. Like all retroviral glycoproteins, ASLV Env precursor is cleaved into the two functional subunits in the golgi apparatus. However, different from all retroviral Env and most class I fusion proteins, the fusion peptide of ASLV Env is found as an internal fusion loop [202]. Similar to influenza HA, the SU and TM of ASLV are linked by an intersubunit disulfide bond, however unlike MoMLV Env, ASLV Env does not contain a CXXC motif [203] and does not contain a proline-rich hinge region [204]. Therefore, an isomerase activity is unlikely for ASLV Env and it has been hypothesized that the harsh condition of a low pH trigger is necessary to break the intersubunit disulfide bond.

Several studies showed that the first trigger for ASLV Env fusion activation is the binding to its cognate cell entry receptor at neutral pH, either TVA or TVB to ASLV-A and ASLV-B, respectively [35]. It was found that receptor binding induces conformational change in the SU subunit, as demonstrated by fluorescent probes and other methods [204]. In addition, receptor interaction led to association to artificial membranes or liposomes at neutral pH, probably by the insertion of the fusion peptide of TM into the target membrane [85]. Whether the receptor dissociates from the fusion complex after or during membrane interaction is still unclear. Nevertheless, the fusion process cannot proceed without the indispensable pH pulse [84, 85]. Indeed, formation of the hemifusion diaphragm was only observed when the membrane-associated fusion complex was treated with a low pH buffer.
In other words, the formation of the hairpin following insertion of fusion peptide seems to require an acidic pH [205]. Based on the principle that the TM conformations are stable and can resist a mild SDS treatment, the different conformational changes in TM were visualized on gel [200, 205, 206].

1.3.4 *Non-canonical pH-dependent mechanism*

A novel pH-dependent mechanism of fusion activation has been found for Ebola virus, by which pH-dependent proteases are required for entry [24]. Other viruses, such as severe acute respiratory syndrome (SARS), MoMLV and the Hendra and Nipah paramyxoviruses, have been also shown to utilize proteases for their entry into host cells [79, 115, 170, 207]. However, whether their fusion activation mechanism is the same as that of Ebola virus remains to be determined.

1.3.4.1 *Ebola virus, requirement for low pH-dependent protease*

Ebola glycoprotein (GP) is a typical class I fusion protein composed of a receptor binding subunit GP1 and a fusion subunit GP2 generated after proteolysis of the GP precursor in the infected cell. One interesting feature of Ebola GP is that, unlike most class I fusion protein, the cleavage is not essential for infectivity [208]. In addition, Ebola GP resembles ASLV Env by that it contains an internal fusion peptide and that the two subunits are linked by a disulfide bond with the absence of an isomerisation motif CXXC [97].

Previous studies have shown that Ebola entry required low pH by using lysosomotropics agents [209]. However, cell-cell fusion was barely detectable following treatment of GP-expressing infectable cells with a low pH buffer [210]. The lack of effect by low pH treatment suggested that acidic pH did not have a direct role in the activation of
Ebola GP. It was later found that the low pH requirement was due to the necessity for pH-dependent proteases, namely cathepsins B and L [24, 210, 211]. The role of proteolytic cleavage in the fusion activation of Ebola GP can explain the infectivity of the uncleaved precursor. The optional cleavage of the precursor fusion protein for infectivity is also observed for Hendra and Nipah viruses [207, 212]. Studies showed that cathepsin cleavage targets the C-terminal region of the Ebola GP1 subunit and generates a 19 kDa fragment from the original 130 kDa subunit. Interestingly, in this form, the fusion activity of GP2 is still inhibited [210]. Thus, this first cleavage event is often referred to as the priming step of the fusion mechanism of Ebola GP. The additional event that leads to complete activation of Ebola GP is still unclear. Possibly, an extra cleavage step mediated by cathepsin L is required [210]. However the nature and the extent of this cleavage remain to be determined. Another possibility is the requirement of other cellular enzymes such as protein disulfide isomerases residing in endosomal and lysosomal compartments [210]. In addition, proteins involved in the modulation of cathepsin L activity may be involved. Another factor adding complexity to the investigation of Ebola entry is that the identity of the receptor(s) is still unknown.

1.4 **Modulation of fusion activation and its inactivation**

The fusion activity of viral fusion proteins must be tightly regulated, and must be activated during infection at the right time and right location. This is not only because the premature triggering causing the usually irreversible conformational change leads to the inactivation of the viral particles, but also because a high fusion activity of the fusion protein at the surface of the infected cells can induce cell-cell fusion, which would be
detrimental to viral fitness. Several factors are involved in the regulation of the fusion activity or modulation of the fusion process such as: subunit interaction, the sequence and conformation of the fusion peptide, the heptad repeats or domains responsible for the hairpin formation, the transmembrane domain, and cytoplasmic tail of the fusion protein.

1.4.1 Clamp release

As mentioned previously, interaction with an inhibitory viral protein or subunit is a redundant modulator of fusion activation. While this interaction is sometimes covalent or non-covalent, the principle underlying this mechanism is the same: to maintain the fusogenic protein or subunit in its pre-fusion state. For class I and class II fusion proteins, the fusion peptide is buried at the subunit interface or at the interface of the protein-protein complexes respectively shielding it from the aqueous environment. Therefore, for the fusion peptide to be inserted into the target membrane, the complex has to dissociate or at least loosen.

For most class I fusion proteins, the clamp is constituted of the surface subunit that interacts with the fusion subunit, through which the conformational rearrangement of the fusogenic subunit is prevented [106]. While the overall mechanism is similar, the trigger that releases the clamp varies. In some cases, such as most retroviral Env, receptor interaction with the surface subunit induces an initial conformational rearrangement in the SU subunit, which further modifies the nature of its interaction with the fusogenic subunit [35, 173]. For example, isomerisation of the disulfide bond linking the two subunits has been shown for some viruses. In other cases, for instance HIV Env, the interaction involves non-covalent interactions between the two subunits [173]. Interestingly, as mentioned previously, for ecotropic MoMLV, it has been shown that cleavage of the SU subunit even
enhances the fusogenicity of the Env [170]. It was hypothesized that the proteolysis of SU liberates the TM subunit to refold and to promote fusion. While shedding of SU in retroviral Env does not always correlate with the extent of fusion, it is generally accepted that in order for TM to form the rod-like structure and insert the fusion peptide in the target membrane, SU-TM has to dissociate to some extent.

In the case of class II fusion proteins, the interaction with the companion protein prevents folding of the fusion protein. One important regulatory mechanism is the processing or maturation of the companion protein by the cellular protease furin. Without the proteolytic cleavage, the fusion process is inhibited and this leads to loss of viral infectivity [116]. For SFV, if p62 is not processed into E2, fusion can still proceed, yet the pH value needed is extremely low, below pH 5.0 as compared to the normally required pH 6.2 [123]. This change in the pH threshold is presumably due to the strength of interaction between the unprocessed or processed p62 and E1. The unprocessed p62 is tightly associated with E1, thus requiring a stronger trigger for dissociation.

This concept of clamp release is further complicated by the fact that several of these interactions have been shown not only to inhibit conformational rearrangement of the fusogenic protein or subunit, but may be required for proper triggering of the latter. This is mostly the case for viral fusion proteins that requires receptor interaction for fusion activation. In this situation, receptor binding allows a positive signal to be sent from the interacting protein or subunit (clamp) to the fusogenic counterpart. For instance, paramyxovirus fusion machinery involves two different glycoproteins, HN that contains the receptor binding domain and interacts with F, which possesses the fusion activity [182]. Interaction of HN with the receptor induces a positive signal to the F protein which mediates fusion. Interestingly, the HN of other paramyxoviruses have been shown to be
able to compensate for the others, suggesting that the mechanism is conserved in the whole paramyxoviridae family. Moreover, the F proteins of some of these viruses have the potential to mediate fusion in the absence of HN, however the fusion activity of F is enhanced in its presence [97]. A similar mechanism was shown for MLV Env as mentioned in section 1.3.1. A study showed that deletion of the receptor binding domain (RBD) in the SU subunit renders Env non-fusogenic and addition of soluble RBD in trans rescues the fusion activity of Env [167]. This suggests that a positive signal is sent to TM upon receptor interaction.

1.4.2 Fusion peptide

Fusion peptides are hydrophobic stretches of residues and they can normally be identified by sequence analysis. The fusion peptide mainly resides at the N-terminus for class I fusion proteins and are found as an internal loop in the case of class II members, and is normally confirmed using mutagenesis [116]. The latter are most of the time stabilized by a disulfide bridge, leaving the loop protruding from the fusion protein, which lies parallel to the viral surface. Class III fusion proteins are very different since the fusion peptide is made of two regions of the protein [130, 213]. In all cases, during insertion into the target membrane, the fusion peptide only interacts with the outer leaflet of the phospholipid bilayer [106].

1.4.2.1 Class I, N-terminal fusion peptide

Analysis of the structure of N-terminal fusion peptides revealed that these are present in solution as a random coil and adopt an α-helical and/or β-fold when interacting
with membranes. HIV Env fusion peptide, for instance, is found as an extended \( \alpha \)-helix in SDS or micelles [214]. However, some other studies reported that it adopts a \( \beta \)-strand structure [215]. These discrepancies may be explained by the variation in the lipid composition used in the different experiments. Indeed, the presence of cholesterol and sphingomyelin in the membrane was shown to favour \( \beta \)-strand formation over the \( \alpha \)-helix [216]. The plasticity of fusion peptide might be important in the fusion process because of the heterogeneity of the cell membranes it requires to fuse with, or the fusion peptide might have to adopt different conformation during the fusion process. For instance, a \( \beta \)-sheet fold may be important during insertion in the target membrane while, in the final stage of the fusion process, interaction with the membrane spanning domain of the protein may require an \( \alpha \)-helical conformation [217]. In addition, it seems that insertion in an oblique manner is thought to be critical for proper interaction with the membrane. Moreover, structural analysis of HA fusion peptide showed that it adopts a kinked structure with charged residues at the tip of the turn [218]. This structure positions the charged residues at the membrane-water interface with the N- and C-terminus inserted in the target membrane. Mutagenesis analysis showed that if the fusion peptide failed to form the kinked structure, it cannot be inserted as deep in the target membrane resulting in reduced fusion activity [219, 220].

The internal fusion peptide of ASLV is found as a hydrophobic loop containing central proline residues and delimited by two cysteines that are thought to form a disulfide bond and stabilize the loop [221]. The disulfide bond, or at least the presence of these two cysteines, seems to be critical for ASLV fusion activity, especially for the lipid mixing stage of fusion [222]. The reason is still unclear, but it is possible that those residues and
the disulfide bond they form are important for the maintenance of the structure, more precisely when the pre-hairpin folds into the hairpin leading to hemifusion. Interestingly, similarly to N-terminal fusion peptides, the fusion peptide of ASLV seems to possess some plasticity depending on its environment [223], suggesting that plasticity of the fusion peptide might be a hallmark of class I fusion proteins. Indeed, the ASLV fusion peptide seems to adopt a β-fold in solution and transforms into a more α-helical structure in the presence of liposomes. In addition, it was shown that low pH causes a deeper insertion of the peptide, and in an oblique angle in the target membrane [223].

1.4.2.2 Class II, fusion loop

The fusion peptides of alphaviruses and flaviviruses are internal fusion loops shielded in the pre-fusion state at the dimer interface. Similar to that of ASLV and Ebola virus, the internal fusion peptide of class II fusion proteins is composed of a loop found at the tip of domain II containing two cysteine residues linked by a disulfide bond at its border [106]. However, unlike the ALSV fusion peptide, they are predicted to have a more rigid structure not affected by the environment. Interestingly, studies have shown that mutations in the fusion peptide domain of SFV can result in the modification of the pH required for fusion and can also modulate the stability of the heterodimer [224-226]. During the low pH fusion activation of SFV fusion proteins, the E1-E2 heterodimer dissociates resulting in the insertion of the fusion peptide of E1 into the target membrane [227]. Interestingly, the insertion of the fusion peptide possibly requires low pH as well [228]. In addition, several studies have shown that low pH is not sufficient, and that the membrane insertion of the E1 fusion peptide of SFV is also dependent on the presence of cholesterol and sphingolipids [127, 229]. Similar cholesterol requirements were reported for the fusion of the flavivirus
TBEV [230]. Analysis of mutants of E1 of SFV cultivated in cholesterol-depleted insect cells showed that the residues found in close proximity of the fusion peptide in the native structure, but not in the fusion peptide itself, are involved in lipid recognition [231, 232]. Consequently, it is possible that the requirement of particular lipids for fusion is not necessarily dictated by the fusion peptide, and could be important at a step prior to fusion peptide insertion into the target membrane [233].

1.4.2.3 Class III, bipartite fusion peptide

The unusual fusion domains of the class III fusion proteins are still not well characterized. Structural and mutational analysis of VSV-G and HSV-1 gB revealed that the fusion peptide is bipartite and composed of two fusion loops linking at the tip of domain I [111, 113, 234]. Class I and II fusion peptides are relatively conserved and typically enriched in small hydrophobic residues such as leucine, isoleucine, alanine and glycine [30]. In the case of class III fusion peptides, their sequence is not conserved even among different herpes viruses [130]. Another striking difference is that they are not necessarily enriched in hydrophobic residues and, depending on the fusion proteins, they do not adopt a conformation that would typically insert into membranes. Instead, residues that were shown to be critical for function of class III fusion peptides are mostly tryptophan and tyrosine residues [213, 235, 236]. Interestingly, aromatic residues such as tryptophan and tyrosine do not have a high tendency for insertion in the lipid bilayer but are normally found at membrane interfaces [237]. Consequently, it is possible that the fusion domains of class III fusion proteins do not insert in the membrane like those of class I and II, yet they possibly interact with the bilayer and cause the destabilisation required for membrane fusion. However, probably due to their complexity, the structure of these bipartite fusion peptides
has not been investigated in the presence of lipids. As a consequence, the structural plasticity is unknown but most likely resembles that of class II fusion proteins since they are internal loops as well.

1.4.3 **Class I fusion protein heptad repeats as antiviral peptides**

During six-helix bundle formation of class I fusion proteins, two important regions of the extracellular domain, or ectodomain, of the transmembrane fusion protein interact tightly together to form the hairpin structure [238]. These are the N and C-terminal heptad repeats (HR) and peptides derived from these regions can efficiently inhibit the six-helix bundle formation supposedly through competition with its intramolecular counterpart [239-242]. HIV heptad repeats have been extensively studied. Studies showed that when peptides derived from the N and C-terminal HR interact they are found as two antiparallel α-helices forming a highly stable trimeric complex of heterodimers [240]. The stability of the heptad repeats interaction originates in part from a salt bridge between a positive arginine residue of the N-HR and a negative aspartate residue of the C-HR. Non-conserved mutations have led to the generation of a non-fusogenic HIV Env [243]. In addition, the hydrophobic interaction at the interface of two interacting heptad repeats is of great importance in the stability of the heterodimers [244, 245].

1.4.4 **Class II fusion protein, antiviral peptides**

Unlike class I fusion proteins, the formation of the trimer of hairpins for class II fusion proteins involves interaction of bulky domains instead of short alpha-helical regions [37]. As a consequence, the design of inhibitory peptides for class II fusion proteins is challenging. However, similar to class I fusion proteins, members of class II must also
undergo conformational change that involves the formation of hairpins to achieve fusion, which offers several alternatives for fusion inhibition. For instance, as described previously, class II fusion proteins bring the viral and the cellular membranes in close proximity by the refolding of domain III against the central core. One study showed that purified domains III of SFV and DV can potently inhibit fusion and infection of alphaviruses and flaviviruses, through binding to the fusion protein and, therefore, preventing the formation of the hairpin [246]. Interestingly, cross-reactivity between genuses was observed, indicating that the interaction is not sequence-specific, but is likely structure-dependent [246]. Another possible target for inhibition of DV E-mediated fusion is a hydrophobic pocket within a flexible hinge region between domain I and II that, during conformational change, drastically modifies its angle. Mutation in this region results in alteration of the pH threshold, indicating that this region is important during fusion activation [247]. Therefore, a small molecule or peptide targeting this region could potentially inhibit the overall conformational change and fusion [247]. In order to investigate the potential use of peptides targeting other important regions involved in the formation of the trimer or in membrane interaction, one study used the DV and WNV E structural data and analysed them with the Wimley-White interfacial hydrophobicity scale [248] to identify sites important for protein-protein and protein-lipid interactions [249]. Their study revealed that peptides analogous to regions of domain II and the stem domain were effective inhibitors of fusion and infection [249]. While more studies are required, these reports indicate that, similar to class I fusion proteins, the conformational change in class II fusion proteins can be exploited by the use of antiviral molecules that inhibit fusion.
1.4.5  Transmembrane domain

Fusion proteins are typically transmembrane proteins and the transmembrane domain plays a critical role in the fusion process. Studies using a modified HA protein where the membrane spanning domain has been deleted and replaced by a sequence for the addition of a GPI anchor, has provided evidence that the transmembrane region is important for full fusion to occur. Indeed, the GPI-HA could associate with the target membrane upon low pH trigger, induce hemifusion and even the formation of small pores in some cases, but not complete fusion [250, 251]. Other groups have also shown that there is a minimal length required for the membrane spanning domain for complete fusion to occur. For instance, deletion of the C-terminal 12 residues of the membrane spanning domain of the HA protein leads to a protein that induces hemifusion but incapable of promoting full fusion [252]. Interestingly, the addition of an arginine residue at the C-terminal position of this truncated HA restored its full fusion potential [252]. It was hypothesized that the addition of the arginine residue at the end of this shortened transmembrane domain allowed it to span completely the membrane via interaction of the positive charge of arginine and the head of the negatively charged phospholipids of the membrane [253]. Strikingly, an arginine residue at the membrane spanning domain is conserved in several retroviral fusion proteins [254].

In many cases, the sequence of the membrane spanning domain is not critical for fusion. This has been demonstrated by swapping the transmembrane domain of other fusion proteins and even unrelated transmembrane proteins [255]. These chimeras were still functional suggesting that the fusion activity remains as long as the transmembrane region completely spans the bilayer. However, it is clear that the membrane spanning domain plays a role in oligomerisation, trafficking, and possibly incorporation of the fusion protein.
onto viral particles. As a consequence, the sequence of the transmembrane region is of importance for some viral proteins for proper folding and oligomerisation which can affect their fusion activity.

1.4.6 **Cytoplasmic tail**

The cytoplasmic tails of viral fusion proteins contains information for the proper trafficking of the protein that targets the glycoprotein to the right cellular localisation in the infected cell. One well studied cytoplasmic tail is that of VSV-G, which contains a motif for its export from the ER to the golgi apparatus [256]. In addition, several fusion proteins contain, in their cytoplasmic tail, endocytosis motifs that are important for their internalization and recycling from the surface of the infected cells [257-259]. The intracellular pathways utilized by these transmembrane proteins are likely to be important for their appropriate glycosylation, which is important for their function, and is critical for the accurate localisation of the fusion protein to the budding site. In addition, some cytoplasmic tails contain regions that interact with the viral structural proteins, such as the capsid [260]. The interaction with the core of the virus can be important to favour the incorporation of the fusion protein onto budding virions.

Importantly, cytoplasmic tails of several fusion proteins are critical regulators for the fusion. This is particularly the case for retroviruses. For instance, several retroviruses, such as MLV, Mason-Pfiser monkey virus (M-PMV), equine infectious anemia virus (EIAV) and others, display an inhibitory mechanism of their fusion activity which involves the C-terminal part of their cytoplasmic tail [261-265]. In this case, the envelope protein with the full length cytoplasmic tail is nonfusogenic. The envelope protein achieves its full fusogenic potential only after the viral protease cleaves off the last 16 amino acid of the
Env cytoplasmic tail, the R peptide, in the budding virions. Interestingly, the fusion inhibitory property of the R-peptide can be transferable to other retroviral envelopes that do not normally contain an R-peptide. The mechanism of inhibition by the R-peptide is still unclear. However, it has been hypothesized that it could be through interaction with the membrane.

Interestingly, it was found that truncation of the cytoplasmic tail of some retroviral Env, that do not possess a R-peptide, can still modulate their fusion activity. For instance, truncation of the cytoplasmic tail of HIV-1, human T-cells leukemia virus-1 (HTLV-1), and simian immunodeficiency virus (SIV-1) Env leads to enhancement of their fusion activity [266, 267]. While the underlying mechanism remains poorly defined, it is thought to occur through change in conformation of the extracellular domain of the protein, known as inside-out signalling [268]. Often, the deleted region that inhibits fusion is characterized by an amphipathic helix that likely interacts with the membrane [269]. Therefore, interaction of the cytoplasmic tail with the membrane frequently correlates with an inhibition of the fusion activity of the fusion protein. It is possible that this interaction increases the stability of the metastable state of the pre-fusion conformation, and, as a result, enhances the amount of energy required to overcome the energy barrier to reach the post-fusion conformation. In any case, one potential reason of this regulatory mechanism used by viruses is to control the fusion activity of the viral fusion protein in the infected cell to avoid fusion of the infected cell with neighbouring cells, which leads to cell death and is detrimental for viral fitness.
1.5 Jaagsiekte sheep retrovirus (JSRV)

In 1825, a farmer in South Africa reported an unknown fatal disease that afflicted several of his sheep, and that he called Jaagziekte; a Dutch word that can be translated into Afrikaans to Jaagsiekte meaning chasing (jaag) sickness (siekte) since the farmer noticed that the sick sheep appeared out of breath as if they had been chased [270]. The affected sheep developed lung tumors, leading to breathing difficulties and accumulation of pulmonary fluid. The disease, also called ovine pulmonary adenocarcinoma (OPA), was then reported in several other countries. The viral etiology of the disease was suggested due to the observation in 1974 of viral particles in sheep lung tumors as well as transmission experiments [271]. These viral particles were later found to have a reverse transcriptase activity in 1976 [272]. The virus, now named Jaagsiekte sheep retrovirus (JSRV), causes great economical losses, and is found throughout the world except in Australia, in New Zealand, as well as in Iceland. However, when OPA was epidemic in Iceland and Kenya, the annual mortality rate caused by JSRV reached up to 50% [270]. In addition, it has been reported that JSRV accounts for 70% of all sheep tumors, with its most famous victim being the first cloned mammal Dolly the sheep [273]. When the disease is not prevalent in a flock, the average incubation period until the appearance of symptoms is around 6-8 months. In experimental conditions, the appearance of tumors in inoculated new born lambs requires 3 to 6 weeks, but can be as quick as 4 to 6 days, suggesting that JSRV encodes a strong viral oncogene [274].
1.5.1  *JSRV* genome and replication strategy

Unique among acutely transforming retroviruses, JSRV is replication-competent, and does not contain a host-derived oncogene. Its genome contains the four main retroviral genes: *gag*, *pro*, *pol* and *env*. The *gag*, *pro*, and *pol* genes are encoded by different independent overlapping open-reading frame with that of the *env* gene partially overlapping the 3’ end of *pol*. As for all retroviruses, the *gag* gene encodes for the matrix (MA), nucleocapsid (NC), and capsid (CA) proteins. The *pro* and *pol* genes encode the three viral enzymes; the protease, reverse transcriptase and integrase [35]. The *pro* gene, in addition to encoding the viral protease, also encodes a dUTPase whose functions is to hydrolyse dUTP to dUMP which, in contrast to dUTP, cannot be incorporated into the newly synthesized DNA strand during reverse transcription that would be otherwise detrimental to the virus [35, 275]. Finally, transcription of the *env* gene generates the only mRNA that is spliced and produces the viral glycoprotein or envelope protein (Env). Intriguingly, JSRV also contains an extra open-reading frame, located in the *pol* gene, termed *orfX*. Sequence comparison showed some similarity with the adenosine A3 receptor [276] but it is unclear if *orfX* generates a protein product.

In 1983, it was found that M-PMV and murine mammary tumor virus (MMTV) capsid antisera cross-reacted with JSRV by Western blot [277]. This, along with its sequence similarity with the reverse transcriptase, led to its classification into the betaretrovirus genera. As all retroviruses, betaretroviruses initiate infection by the recognition of a cellular receptor at the surface of the target cell, and eventually by the fusion of its viral membrane with that of the host cell (for a more complete overview [35]). The reverse transcriptase then initiates the synthesis of the first DNA strand using the viral RNA genome as a template and a specific tRNA as a primer. Concomitantly, the viral RNA
used as a template is degraded by the RNaseH activity of the reverse transcriptase. However, an RNase-resistant region remains and is used to prime the synthesis of the second DNA strand. The complex migrates to the nucleus where the integrase performs the integration of the provirus into the host genome. Simple retroviruses, such as JSRV, require the host cell to proliferate because the transport of the pre-integration complex requires the disruption of the nuclear membrane that occurs during cell division. After integration, the viral proteins are then expressed and assembled, and the virions then bud from the plasma membrane where the viral particles acquire their viral membrane and the viral glycoproteins spanning the lipid bilayer. The betaretroviruses are different from other retroviruses in the fact that the assembly of the viral core takes place in the cytoplasm of the host cell instead of occurring at the plasma membrane. The assembled core then migrates to the surface and buds from the plasma membrane. Following a maturation step that involves the cleavage of viral proteins by the viral protease, the newly infectious viral particle can now infect another cell and the cycle starts again [35].

1.5.2  *Enzootic nasal tumor virus, a related oncogenic betaretrovirus*

Enzootic nasal tumor virus (ENTV) is betaretrovirus of sheep and goat that induces contagious nasal adenocarcinoma [278-281] (reviewed in [282]. ENTV is closely related to JSRV and shares around 95% overall amino acids similarity, and its genome also contains *orf-x*, yet the open reading frame is terminated prematurely by the presence of two stop codons [278]. In addition, the Env of ENTV shares around 89% sequence identity with that of JSRV and the membrane spanning domain and the CT being the most divergent regions [278] (Fig. 1.5). Interestingly, ENTV Env has a relatively long CT of 47 amino acid residues, which is similar to JSRV Env CT that is composed of 44 residues. Both viruses
use the same entry receptor, hyaluronidase-2 (Hyal2), as a cell entry receptor, yet they infect and replicate in different cell types [283]. While JSRV induces lung tumors derived from type-II pneumocytes, ENTV induces tumors arising from the transformation of nasal secretory epithelial cells, causing cranial deformation and eventually death. It is now generally believed that the differential tropism of JSRV and ENTV is mainly dictated by their respective LTR activity in the different cell type [284, 285].

**Fig.1.5 Sequence alignment of sheep retrovirus envelope proteins.** Amino acid sequence alignment of JSRV-7 (accession number AAK38688), ENTV-1 (accession number Y16627) and endogenous JSRV en5F16 (accession number AF136224). Cyttoplasmic tail region is colored in green, membrane spanning domain labelled in blue and all the different residues in the TM ectodomain or the SU subunit are identified by a red box.
1.5.3  *Endogenous JSRVs in the sheep genome*

One very surprising feature of JSRV pathology in sheep is that even though JSRV induces massive tumor formation, there is very little or no immune response raised against JSRV in infected sheep, and studies have been unsuccessful to detect antibodies against JSRV particles [274]. One possible reason for the apparent tolerance of sheep to JSRV is the presence of multiple copies of endogenous retroviruses in the sheep genome, collectively called enJSRVs, highly related to JSRV [286]. In fact the LTR and *gag* regions of some enJSRVs can reach up to 95% sequence identity to their exogenous counterpart. These endogenous retroviruses are quite recent, and some seem to have been fixed in the genome as late as 4-10 million years ago, after sheep and goats have diverged, this process is probably still ongoing [287]. As a consequence, some of these endogenous retroviruses still contain intact retroviral genes, yet they are unable to replicate. The sequences of several complete genomes of enJSRV have been reported [287]. One of them, enJS59A1, has a major deletion in the *env* gene and another one, enJS5F16, contains a truncated version of the *pol* gene. In addition, the endogenous JSRV clone enJS56A1, contains in its genome all the structural retroviral genes but its *gag* gene is defective. Interestingly, the non-oncogenic Env proteins of JS5F16 and others are well conserved and highly related to the exogenous JSRV except for the cytoplasmic tail (Fig. 1.5).

Retroviral mRNA of these viruses can be detected in almost all tissues [288]. However, the viral proteins of these endogenous retroviruses are particularly expressed in the genital tract of the ewe [289]. There have been suggestions that the Env of these endogenous retroviruses may serve as the syncytin proteins in human in the formation of the syncytiotrophoblast but this remains to be addressed [290]. In any case, the expression
of these viral proteins in the newborn lamb may have a profound effect in the development of tolerance to JSRV since these antigens will be recognized as self.

However, there might be beneficial aspects for the expression of viral proteins from these endogenous retroviruses. For instance, receptor interference could counteract the exogenous JSRV since enJSRVs and JSRV all use a common receptor, Hyal2. Another restriction mechanism unique to enJSRVs is the inhibition of a late stage of the replication of the oncogenic JSRV in cells expressing the defective gag protein of enJS56A1 [291]. Interestingly, the latter gag protein contains a single mutation near the N-terminus of the protein. This causes modification of the normal trafficking of gag and its accumulation in aggresomes inhibiting viral assembly [292]. More interestingly, it was shown that this effect is transdominant. As a result, in cells infected with the oncogenic JSRV, the expressed defective gag protein enJS56A1 interacts with the oncogenic JSRV gag protein and alters the trafficking of the latter leading to inhibition of viral assembly and release [291]. However, the role of this mechanism in JSRV pathogenesis remains to be determined.

1.5.4 Hyaluronidase-2 functions as JSRV cell entry receptor

The identification of JSRV cell entry receptor was initially possible because of the ability of JSRV Env to pseudotype efficiently retroviral and lentiviral vectors [293, 294]. Using MLV vectors encoding the human placental alkaline phosphatase (AP) and harbouring JSRV Env, different cells lines were analysed for transduction of JSRV pseudotypes. This analysis revealed that sheep and human cells are permissive to JSRV pseudovirions, yet rodent cells, including mouse, rat, and hamster cells, are resistant to infection. Consequently, the inability of JSRV to infect rodent cells allowed the screening
of human genes that when expressed in hamster cells can confer JSRV pseudovirion infection. The gene of JSRV Env receptor was found to be localised to human chromosome 3p21.3 [293]. This region is known to encode three members of the hyaluronidase family; hyaluronidase-1 (Hyal1), Hyal2 and Hyal3 [295]. Further analysis indicated that expression of human hyaluronidase2 (Hyal2) is necessary and sufficient to confer susceptibility of hamster cells to JSRV transduction [296]. In addition, as expected, ovine Hyal2 also functions efficiently as a receptor and, in contrast, the mouse ortholog functions poorly while Hyal2 of rat exhibits intermediate functionality [283, 296]. The region of interaction with JSRV is rather complex, but mainly involves the central third portion of the receptor [297]. Hyal2 was also found to serve as a cell entry receptor for ENTV and pseudotypes harbouring enJSRVs Env [283, 298]. However, ENTV pseudovirions exhibit a host range that is restricted to sheep and some human cell lines, whereas retroviral vectors bearing JSRV Env can infect most human, monkey, dog, cow, and rabbit cells [283]. In addition, expression of human or sheep Hyal2 in rat cells confers susceptibility to JSRV vectors, yet does not allow ENTV vectors transduction, suggesting that ENTV entry involves additional cellular factors other than the expression of Hyal2 [283, 299].

Hyal2 was originally thought to have a lysosomal localisation, using a Hyal2 construct fused to GFP at the carboxy terminus [300]. However, Hyal2 was later on found to be a GPI-anchored protein expressed at the cell surface [296, 301]. The mislocalisation of the former construct can be explained by the cleavage of the GFP portion of the fusion protein during the addition of the GPI anchor to Hyal2. Intriguingly, Hyal2 hyaluronidase activity is very low compared to another member of the hyaluronidase family Spam1 (around 400 folds less), and exhibits an acidic pH optimum [302, 303]. As a result, the surface expression and the acidic pH optimum of Hyal2 render the reconciliation between
localisation and function difficult, and the role of Hyal2 remains unclear. However, one study reported that the interactions between Hyal2, CD44, and the Na+/H+ exchanger (NHE) protein in lipid rafts, create an acidic microenvironment that activates Hyal2 hyaluronidase activity [304]. Moreover, the Hyal2 gene is localised in the 3p21.3 locus, a region that is frequently deleted in lung, breast, and several other cancers making Hyal2 a putative tumor suppressor [305, 306]. A potential role for Hyal2 in cell transformation was shown to be mediated by its interaction with a receptor tyrosine kinase, and will be described in more details below.

1.5.5  *JSRV and ENTV oncogenesis*

The relationship between retroviruses and oncogenesis is known for now more than 100 years with the isolation of avian erythroblastosis virus (AEV) in 1908 from chicken developing spontaneous erythroleukemia (Ellerman, Bang 1908). Several other retroviruses were also found to induce leukemia, sarcoma, and carcinoma in a wide variety of animals. Human T-cells leukemia virus-1 and -2 (HTLV-1 and HTLV-2) are thus so far, the only known viruses capable of directly inducing cancer in humans. Oncogenic retroviruses can promote tumor formation in a wide variety of ways and have been typically classified into acute and non-acute transforming retroviruses based on the rapidity by which they can form tumors. JSRV and ENTV are two acutely transforming retroviruses that induce tumors in sheep and goats by an unusual mechanism.

1.5.5.1  **Oncogenic retroviruses**

Studies on oncogenic retroviruses have established the foundation of the oncogenesis field with the discovery of viral oncogenes (v-onc) and the critical
understanding that they were derived from the host genome [307]. This acquisition of a cellular proto-oncogene (c-onc) is called oncogene capture. The resulting virus contains in its genome the c-onc, which normally replaced or disrupted viral genes that are important for replication. Consequently, the virus is often replication-defective and requires helper viruses to provide in trans the missing viral genes critical for replication. Those retroviruses containing an oncogene derived from the host cell can induce rapidly the formation of tumors of polyclonal origins and are classified as acutely transforming retroviruses. In contrast, some retroviruses induce tumor formation in an extended time period and are termed nonacutely transforming retroviruses. In this case, the viruses do not contain an oncogene derived from the host cell and they can replicate on their own. Instead, they promote tumor formation by activating cellular proto-oncogenes near the integration site of the virus, a process termed insertional activation [307]. Additionally, some other oncogenic retroviruses induce tumor formation by the expression of some viral genes. For instance, HTLV promotes leukemia in a small proportion of infected individuals by the expression of Tax and HBZ, transcription factors encoded on the antisense strand that induce cell proliferation and the transcription of cytokines and cytokine receptors [308]. Interestingly, another mechanism is exemplified by retroviruses such as avian hemangioma virus and spleen focus-forming virus, which are capable of promoting cell proliferation of particular cell lines and/or in animals by the expression of the native Env or a truncated form of Env respectively [309, 310].

Inoculation of newborn lambs with purified JSRV particles can induce tumor formation in less than two weeks [311]. Therefore, JSRV can be considered an acutely transforming retrovirus, yet its genome does not contain a host derived oncogene and it is a
replicative virus. The Orf-X present in JSRV genome was suspected at first to be involved in JSRV-mediated transformation. However, an implication in oncogenesis of this Orf-X was rapidly discredited since it is not present in ENTV genome, it is found in the genomes of endogenous non-oncogenic JSRVs and its disruption does not affect JSRV oncogenesis [312]. Instead, the envelope proteins of JSRV and ENTV, in addition to mediating cell entry and fusion, also act as active oncogenes that can promote tumor formation in vivo [313, 314] and induce transformation of cultured cells [312, 315-317].

1.5.5.2 JSRV Env-mediated transformation

It is now clear that the cytoplasmic tail is critical for JSRV and ENTV Env-mediated transformation because the replacement of JSRV Env CT by that of other retroviruses abolishes the transformation activity of the protein [315, 316, 318, 319]. However, it is still unclear and controversial whether the CT is sufficient for JSRV Env-mediated transformation [320, 321]. Importantly, a YXXM motif (Y: tyrosine, X: any amino acid, M: methionine), a putative PI3K/p85 binding motif, is found in both JSRV and ENTV Env CT, but is absent in those of enJSRVs. Mutation of the tyrosine residue of the motif was shown in some systems to abolish the transforming activity of Env [318, 322, 323] but was found in others to predominantly reduce it [315, 316, 319]. Therefore, the YXXM motif is important during transformation but its involvement is probably cell-type dependent and varies depending on the experimental procedure. In addition, the motif is possibly important for the modulation of Env expression, trafficking or conformation of the cytoplasmic tail.

The role of the SU subunit in cell transformation is still unclear and is probably cell type-dependent. In rodent cells, in which mouse Hyal2 is not functional as a receptor
for JSRV and ENTV, there was a report that showed that SU is critical for transformation [324] and other studies suggest that SU is not involved [301, 320]. Because mouse Hyal2 is inactive as a receptor, it is still unclear how the SU subunit could be involved in transformation of rodent cells [283]. Interestingly, transformation of the human bronchial epithelial cell line, BEAS-2B, seems to involve the interaction between human Hyal2 and the SU subunit [317]. In this case, Hyal2 acts as a tumor suppressor by binding and preventing the activation of a receptor tyrosine kinase, Récepteur d’origine nantais (RON). Env and Hyal2 interaction results in the degradation of Hyal2 and consequently allows dimerization and activation of RON in the absence of its natural ligand, thereby leading to transformation [317]. However, whether other regions of Env are involved in BEAS-2B transformation remains to be determined.

There are three major pathways by which JSRV Env has been shown to mediate cell transformation and the contribution of each one of them appears to be cell-type dependent. The first one and possibly most important is the activation of the PI3K-dependent or independent Akt pathway. The second one is the Raf-MEK-MAPK pathway and the last one was described above and is the RON-Hyal2 pathway [325].

The PI3K-Akt pathway is operative in rodent, chicken and canine cell lines (NIH 3T3, 208F, CEF, DF-1 and MDCK cells) The involvement of this pathway in JSRV-induced cell transformation is supported by Akt phosphorylation and increased activity in transformed cells derived from different cell lines [316, 318, 319]. In addition, the PI3K inhibitor, LY294002, was shown to inhibit Akt phosphorylation and reverse the transformed phenotype in rodent cells [319]. Although a model where there is activation of the PI3K pathway through recruitment of the PI3K/p85 regulatory to JSRV Env YXXM motif is attractive, no interaction was found between the two proteins and phosphorylation
of the tyrosine of the YXXM could not be observed [319]. By using dominant negative forms of p85 or cells derived from p85 knockout mice, one report suggested that Akt can be activated in a PI3K-independent manner and did not affect JSRV transforming activity [326]. However, investigation of the signalling pathways activated in JSRV-induced sheep lung tumors and chicken fibroblast cells (DF-1) revealed that Akt activation is not involved and Akt phosphorylation was not detected. Interestingly, Akt phosphorylation was observed in ENTV-induced nasal tumors [327].

The second pathway, the Raf-MEK-MAPK pathway, was shown to be involved in JSRV-induced transformation by the inhibition of transformation of NIH 3T3 cells in the presence of MEK-1 inhibitor, PD98059, or a H/N-Ras inhibitor, FTI-277, suggesting that the Ras-Raf-MEK-ERK pathway might be important [328]. In addition, ERK1/2 phosphorylation was observed in JSRV-induced sheep lung tumors [328]. Surprisingly, ERK1/2 phosphorylation was not detected in NIH 3T3 and 208F transformed cells [319, 328]. The reason why Erk1/2 are not found to be phosphorylated might reside in a cross-talk between the ERK and p38 pathways. Interestingly, an inhibitor of p38, SB203580, was shown to enhance JSRV-induced cell transformation in NIH 3T3 cells and increase ERK1/2 phosphorylation indicating that the p38 pathway might be acting as a negative regulator of JSRV mediated transformation [328].

One major question remaining is how JSRV and ENTV Env induce tumor formation. Recently, RON has been shown to interact with JSRV Env and the CT is involved in RON activation state [321]. However, the significance of this interaction in transformation remains to be determined. In addition, involvement of HSP90 in JSRV transformation was also reported, yet no interaction between the two proteins was detected.
Consequently, it is likely that JSRV- and ENTV-induced cell transformation involves a yet unidentified cellular factor that interacts with Env.

1.5 Rationale and hypothesis

Being transforming retroviruses, it is plausible that JSRV and ENTV may have evolved mechanisms to negatively regulate their fusion activity in order to prevent syncytium formation caused by the fusion of the Env-expressing transformed cells with Hyal2-expressing neighboring cells, which would be detrimental to viral fitness. In addition, a growing number of retroviruses are being reported to require low pH for entry and fusion. Therefore, we hypothesized that JSRV and ENTV necessitate acidic pH for fusion and infection. The goal of my project is to determine the entry pathway and fusion mechanisms of JSRV and ENTV, and to understand how these processes are regulated.
CHAPTER 2

FUSOGENICITY OF JAAGSIEKTE SHEEP RETROVIRUS

ENVELOPE PROTEIN IS DEPENDENT ON LOW pH AND

IS ENHANCED BY CYTOPLASMIC TAIL TRUNCATIONS

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2.1 Preface to Chapter 2

When we first started to work on fusion and entry of Jaagsiekte sheep retrovirus, the role of the envelope (Env) protein in oncogenesis was extensively studied, yet nothing was known about how it mediates entry of the virus into the host cell. In addition, there was no fusion assay available to investigate how JSRV Env induces fusion. Consequently, the first step was to design a new method that would enable us to observe and quantify fusion events mediated by Env.

One way to study viral glycoprotein-mediated fusion is to mimic the viral-cell fusion by a cell-based assay where one cell expresses the viral fusion protein (the substitute for the virus) and the other cell harbours the receptor at its surface (represents the target cell). Additionally, because most retroviruses are thought to be pH-independent and require receptor binding for fusion, we attempted to create a fusion assay using JSRV permissible cells and cells overexpressing the receptor. On the other hand, this generalised idea was being more and more challenged by the discovery that many retroviruses require low pH for entry. Therefore, we wanted to investigate the possibility that JSRV Env requires acidic conditions to mediate fusion, a mechanism that could be important in the modulation of the fusion activity of this oncogenic Env.

Finally, one way several retroviruses regulate their fusion activity is by the removal of a short a.a. sequence at the C-terminus of the Env cytoplasmic tail by the viral protease in the budding virions. This activation mechanism prevents undesirable fusion events between infected cells expressing the Env at their surface and neighbouring cells expressing the receptor. Thus, we decided to study the effect of C-terminal truncation mutants of JSRV Env on fusion activity using our newly-developed assays.
2.2 Abstract

Jaagsiekte sheep retrovirus (JSRV) envelope (Env) is an active oncogene responsible for neoplastic transformation in animals and cultured cells. In this study, we used syncytium induction and fluorescence-based cell fusion assays to investigate JSRV Env fusion and its modulation by the cytoplasmic tail (CT). We found that JSRV Env induced syncytia in cells overexpressing the receptor for JSRV and that low pH was required for this process to occur. Fusion kinetics studies revealed that cell-cell fusion by JSRV Env at neutral pH was poor, taking up to a day, in sharp contrast to fusion at low pH which peaked within 2 min following a low pH trigger. Deletion of the C-terminal seven or sixteen amino acids of the JSRV Env CT had no or little effect on fusion; yet additional truncation toward the membrane-spanning domain (MSD), resulting in mutants retaining as little as one amino acid of CT, led to progressively increased syncytium formation at neutral pH that was further enhanced by a low pH treatment. Notably, the severely truncated mutants showed elevated levels of surface subunits in culture medium, suggesting that the CT truncations resulted in conformational changes in the ectodomain of Env that impaired surface subunit associations. Taken together, this study reveals for the first time that the fusion activity of JSRV Env protein is dependent on low pH and is modulated by the CT whose truncation overcomes, at least partially, the low pH requirement for fusion, and enhances the Env fusion activity and kinetics.
2.3 **Introduction**

Fusion of enveloped viruses with host cells is a fundamental process essential for viral replication and productive infection. It occurs either at the plasma membrane at neutral pH or in the intracellular compartments where an acidic pH is required [30]. Retroviral fusion is generally believed to be triggered by specific interactions between the viral Env and its cognate cell-entry receptor, and coreceptors in some cases. Upon receptor binding, the envelope (Env) protein undergoes a series of conformational changes on the surface (SU) and transmembrane (TM) subunits, leading to insertion of the Env fusion peptide into the cell membrane and subsequent formation of trimers of hairpin structures (also referred to as six-helix bundles) that complete virion-cell fusion [177]. Recent studies indicate that for five retroviruses, ecotropic murine leukemia virus (ecotropic-MLV) [72, 330], avian sarcoma and leukosis virus subgroup A and B (ASLV-A and B) [200, 331], mouse mammary tumor virus (MMTV) [332, 333], foamy virus (FV) [334], and equine infectious anemia virus (EIAV) [335, 336], fusion occurs in endosomes or other intracellular compartments, and requires an acidic pH. Moreover, depending on viral strains and cell types, some retroviruses may use both pH-dependent and pH-independent pathways for fusion and cell entry [72, 330, 337].

The fusion activity of retroviral Env is controlled by viral and host factors at multiple levels. In addition to the universal cleavage of a precursor Env into SU and TM subunits by cellular proteases in the Golgi complex that converts the Env to a metastable state, a portion of the CT of many retroviruses is removed by viral protease cleavage after virion assembly [35]. For example, the Env protein of Moloney MLV (MoMLV) [263, 264, 338, 339], gibbon ape leukemia virus (GALV) [340] and several other simple retroviruses
contain a ~16-amino-acid (aa) segment at the C-terminus of CT, termed the R peptide, that modulates Env fusogenicity. The R peptide must be removed to fully activate the Env fusogenicity after new viral particles are released [261-265]. This mode of regulation by the Env CT is believed to be important for viral fitness, because it potentially prevents syncytium formation in infected cells while activating Env on newly assembled viral particles so that they are competent to infect naïve host cells.

JSRV is a simple betaretrovirus that causes a contagious pulmonary adenocarcinoma (OPA) in sheep [343]. Inoculation of concentrated JSRV virions in newborn lambs induces rapid lung tumor formation in as little as two weeks [311], showing JSRV is an acutely transforming retrovirus. It is now well established that the Env of JSRV is an active oncogene that is responsible for JSRV oncogenesis [296, 312, 313, 315-317, 344, 345]. Of particular interest, the CT of JSRV Env is essential for cell transformation, as replacement of this domain with that of other retroviruses completely abrogates cell transformation [318, 319]. The JSRV Env CT is a forty-four-aa segment, relatively long compared to other simple retroviruses, and harbors several sequence motifs and residues known to be crucial for cell transformation and possibly intracellular trafficking [325]. Importantly, the N-terminal eighteen-aa segment of its CT is predicted to form an amphipathic alpha-helix structure, the hydrophobic side of which has a tendency to interact with cellular membrane [322]. Exactly how this structure might modulate cell transformation and other functions of JSRV Env, including fusion, is currently unknown.

The receptor for JSRV has been identified as hyaluronidase-2 (Hyal2), a glycosylphosphatidylinositol (GPI) -anchored cell surface protein [296]. Hyal2 belongs to the hyaluronidase gene family yet interestingly has a low hyaluronidase activity which normally degrades the hyaluronic acid present in the extracellular matrix of cells [296, 300,
A recent study suggests that the hyaluronidase activity of Hyal2 is not associated with the receptor activity [303]. JSRV infects cells derived from many species, including human, ovine, bovine, and canine, but notably not from rodents, and this host range correlates with the receptor activities of Hyal2 isolated from these species [283, 293, 296, 347].

While the transforming properties of JSRV Env have been studied extensively, its fusion characteristics have not been investigated. In this study, we developed syncytium induction and cell-cell fusion assays to use with JSRV Env, and performed a series of experiments to test the hypothesis that JSRV Env-mediated fusion may require acidic pH and be negatively regulated by its relatively long CT. Our results provide evidence that JSRV entry is pH-dependent and indicate that JSRV Env employs multiple control mechanisms to regulate its fusion activity.

2.4 Materials and Methods

Env constructs. The parental JSRV Env gene derived from the JS7 strain [348], was tagged with a FLAG sequence at the N-terminus of SU, and was initially cloned into a MoMLV LTR-driven expression vector, pSX2neo (the resulting construct was referred to as pSX2neo-FLAG-Jenv [319]). In the present study, the Env-coding region was subcloned into pClneo (Sigma, St. Louis, MO), a CMV-driven expression vector, in order to achieve a higher level of expression in 293 and 293T cells (the resulting construct was referred to as pClneo-FLAG-Jenv). To create the JSRV Env truncation mutants, PCR was carried out using a common upstream primer, 5’-GCCTGGTATGATGAAACTGC-3’, paired with the
individual unique downstream primers listed below to amplify the TM subunits of truncation mutants:

CT608, 5’-GCATAGATCTTCACCTCTCTTTATTTTTAAAAGC-3’;
CT599, 5’-GCATAGATCTCTAAAGATGTTGGTGCTGTA-3’;
CT589, 5’-GCATAGATCTCTATTTTCATATGCAGCATTTC-3’;
CT574, 5’-GCATAGATCTTCACATGCCACGAACGAGGCAAGG-3’;
CT572, 5’-GCATAGATCTTCACGAACGAGGCAAGGAAA-3’;
CT571, 5’-GCATAGATCTTCAACGAGGCAAGGAAATATAAG-3’.

The PCR products were either directly cloned into a pCIneo-FLAG-Jenv backbone, or were first shuttled into the pSX2neo-FLAG-Jenv vector and subsequently into the pCIneo-FLAG-Jenv vector. The 10A1 amphotropic Env-expressing vector was generated by cloning the 10A1 Env-encoding sequence from pSX2 [349] into the pCIneo vector (referred to as pCIneo-10A1). The VSV-G expression vector (pMD.G) has been previously described [350].

**Retroviruses and cell lines.** The GFP-encoding retroviral pseudotypes bearing different Env proteins were produced by co-transfection of 293T cells with plasmids encoding individual Env or VSV-G proteins, a packaging plasmid encoding the MoMLV gag-pol (pCMV-gag-pol-MLV), and a transfer vector encoding GFP (pCMV-GFP-MLV) (the two latter plasmids were kind gifts of François-Loïc Cosset, Lyon, France). The HIV-1 lentiviral vector encoding GFP was generated by co-transfection of 293T cells with pMD.G [350], pCMV-HIVΔ8.2 [350], and pHIV-eGFP (a gift from Eric Cohen, Institut de Recherches Cliniques de Montréal, Canada). Viral pseudotypes were harvested 48 to 72 h
post-transfection, were filtered through 0.45 μm-pore-size filters or centrifuged at 2,500 g to remove the cell debris. The Hyal2-encoding retroviral vector was produced from a PT67/LH2SN producer cell line [296].

293/LH2SN and HTX/LH2SN were generated by infection of 293 or HTX cells with PT67/LH2SN viral stocks in the presence of 5 μg/ml polybrene (Sigma), similar to that of the 3T3/LH2SN as previously described [296]. The 293T/GFP cells were generated by infection of 293T cells with the GFP-encoding HIV-1 vectors bearing VSV-G, followed by ring cloning and flow cytometry to confirm GFP expression. All cells were grown in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5 or 10% fetal bovine serum, and were maintained in 37°C in a 10% CO₂-air atmosphere at 100% relative humidity.

**Antibodies and reagents.** The anti-FLAG monoclonal antibody, EZview™ Red anti-FLAG affinity gel and the secondary anti-mouse IgG coupled to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were purchased from Sigma (St. Louis, MO). Anti-human IgG coupled to FITC was purchased from DAKO Cytometry (Glostrup, Denmark). JSRV SU (JSU) fusion protein (JSU-hFc) for detection of cell surface receptor levels was produced and purified as described previously [301]. The fluorescent dye 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR) and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). FuGENE HD was purchased from Roche (Indianapolis, IN), and Bafilomycin A1 (BafA1) was purchased from Sigma (St. Louis, MO). Pro-Mix [³⁵S] cell labeling mix was purchased from Amersham (Buckinghamshire, England).
Syncytium induction assay. Cells seeded in 6-well plates were transfected with 2 μg of plasmid DNAs encoding each individual Env plus 0.5 μg of plasmid encoding GFP (to monitor transfection efficiency) using the calcium-phosphate method for 293, 293/LH2SN cells or FuGENE HD (Roche, Indianapolis, IN) for the other cell lines. Medium was changed 6-9 h following transfection, and syncytium formation was determined 12-24 h post-transfection under a light microscope. The numbers of syncytium formation per field and the numbers of nuclei per syncytium were quantified, and were averaged from at least four different fields. Cells were subsequently treated with phosphate buffered saline (PBS)-10mM HEPES-10 mM MES (Sigma) pH 5.0 for 1 min, syncytium formation was evaluated under microscope over a period of 5 min to 24 h.

Cell-cell fusion Assay. Effector 293T/GFP cells were transfected with 2 μg of DNA encoding individual Env using Lipofectamine 2000 (Invitrogen). 12 to 24 h post-transfection, cells were washed with Hank’s Balanced Salt Solution (5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 5.6 mM Glucose, 137 mM NaCl, pH 7.4), then detached using PBS containing 5 mM EDTA. Target cells expressing an endogenous Hyal2 or overexpressing human Hyal2 were labeled with 3.5 μM CMTMR (Invitrogen) in media without serum at 37°C for 30 min, then were incubated at 37°C for 30 min in fresh media containing serum, followed by 3 washes with regular media. Effector cells and target cells were then plated onto 24-well plates at a ratio of 1:1 or 1:3, depending on experiments, and were co-cultured at 4°C or 37°C for 1 h. Cells were then treated with PBS-10 mM HEPES and 10 mM MES pH 7.4 or pH 5 for 1 min, and were incubated with normal growth medium at 37°C, or different temperatures, for various periods of time. Cell-
cell fusion was examined under a fluorescent microscope (Carl Zeiss, Goettingen, Germany) and photographed. Alternatively, cells were washed with PBS, detached using 0.05% trypsin-0.02% EDTA (Invitrogen), and analyzed by flow cytometry using FACSCalibur (BD Bioscience, Mississauga, ON, Canada).

**Determination of JSRV Env and Hyal2 surface expression by flow cytometry.** Cells were washed with Hank’s Balanced Salt Solution, detached using PBS plus 5 mM EDTA, and resuspended in wash buffer (PBS plus 2% FBS). $5 \times 10^5$ cells were incubated on ice with anti-FLAG antibody for 1 h, washed twice with wash buffer and incubated with anti-mouse IgG coupled to FITC for 293 cells or PE for 293T-GFP cells for 1 h. Cells were then washed and analyzed by flow cytometry. Hyal2 expression on the cell surface was analyzed similarly except that cells were incubated with purified JSU-hFc fusion protein for 4 h on ice, followed by incubation with anti-human IgG Fc antibody coupled to FITC [301].

**Retroviral vector transduction and Bafilomycin A1 (BafA1) treatment.** $10^5$ cells seeded in 12-well plates on day 0 were pre-treated with 10 or 25 nM BafA1 on day 1 for 2 h, followed by transduction with JSRV/MoMLV pseudotypes in the presence of 5 μg/ml polybrene plus 10 or 25 nM BafA1 at 37°C for 6 h. Non-internalized viral particles were inactivated for 1 min with citrate buffer (40 mM sodium citrate, 10 mM KCl, 135 mM NaCl, pH 3.15). Cells were washed 3 times with PBS then grown for an additional two days before GFP titers were determined by flow cytometry.
**Metabolic labelling.** 293T cells were transiently transfected with plasmids encoding JSRV Env using the calcium-phosphate co-precipitation method. 24 h post-transfection cells were starved in DMEM without cysteine and methionine (Invitrogen) for 30 min, pulse-labeled with 62.5 µCi mixture of Cysteine plus Methionine (Amersham) for 1 h, followed by a chase-labeling for 4 h in the presence of complete growth medium. Cells were then washed with cold PBS once, and lysed in lysis buffer (PBS containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) in the presence of 10 µg/ml aprotinin (Sigma), 10 µg/ml leupeptin (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). Env in cell lysates and in the culture medium were immunoprecipitated using anti-FLAG beads, and the proteins in the immune complexes were resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, the dried gels were submitted to autoradiography and band intensities quantitated using Quantity One (Bio-Rad, Hercules, CA) image analysis software.

**2.5 Results**

**JSRV Env induces syncytium formation at low pH**

We first sought to determine if JSRV Env induces syncytium formation in cells overexpressing human Hyal2, a standard fusion assay not yet applied to JSRV Env. 293/LH2SN cells were transfected with plasmids encoding JSRV Env, 10A1 amphotropic MLV Env, VSV-G, or none, along with a plasmid encoding GFP as a transfection control. 12-24 h post-transfection, cells were examined for possible syncytium formation under microscopy, then exposed to either pH 7.4 or pH 5 solutions and again examined for syncytia. VSV-G mediated fusion has been shown to be pH-dependent while negative...
control amphotropic 10A1 MLV Env does not induce cell fusion at acidic or neutral pH [72, 351]. At neutral pH, no syncytium formation was observed in any of the transfected cells (Fig. 1A, upper panels). By contrast, a 1 min incubation at pH 5 resulted in substantial levels of syncytium formation for JSRV Env (Fig.1A, lower panels). The JSRV Env-induced syncytium formation appeared at approximately 10 minutes after the low pH trigger, compared to that of VSV-G that induced massive syncytia within 5 min of low pH treatment. The difference between JSRV Env and VSV-G was not distinguishable after 30 min of low-pH treatment, as virtually all cells fused for both proteins. These data clearly demonstrate that JSRV Env induces syncytium formation in 293 cells overexpressing human Hyal2 and that a low pH treatment is required for this process to occur.

We next examined syncytium induction in 293T and 293 cells that express an endogenous level of human Hyal2. These two cell lines are highly susceptible to JSRV vector transduction, although the titers of JSRV in these cells were approximately five-fold lower than that in 293/LH2SN cells (data not shown). While syncytium formation was occasionally observed in these two cell lines by JSRV Env at pH 5 but not at pH 7.4, the efficiency was extremely low, as reflected by the very low numbers of syncytium formation (~2 per dish) and the small sizes of syncytia (~4 nuclei per syncytium) (data not shown). To determine if this was a cell-type specific effect, we next performed syncytium formation assays in human HTX and HTX cells overexpressing human Hyal2 (referred to as HTX/LH2SN cells hereafter). Even more surprisingly, only 2-3 syncytia per field were observed in HTX/LH2SN cells at pH 5 (Fig. 1B, lower panels), and none was detected in HTX cells (data not shown) even though they are as susceptible as 293 cells to JSRV vector transduction [293]. In vitro binding assays showed the Hyal2 expression level on the
surface of HTX/LH2SN cells was equivalent to that in 293/LH2SN cells (Fig. 1C), suggesting that the low efficiency of syncytium formation by JSRV Env in HTX/LH2SN was not due to a difference in Hyal2 expression. While the transfection efficiency of HTX/LH2SN cells (Roche FuGene HD method, ~60%) was not as high as that of 293/LH2SN (~100%), this resulted in a small reduction in the JSRV Env expression in HTX/LH2SN cells relative to that in 293/LH2SN cells (data not shown). Furthermore, VSV-G invariably induced large and comparable numbers of syncytia in both HTX/LH2SN (Fig. 1B) and HTX cells (data not show), with efficiencies similar to that in 293/LH2SN cells, suggesting that the low syncytium formation efficiency by JSRV Env in HTX/LH2SN cells was not solely due to its relative low transfection efficiency. Therefore, syncytium formation by JSRV Env is not only determined by the levels of Hyal2 expression in the indicator cells but appears to be affected by the cell lines used in the experiments.

Development of a cell-cell fusion assay for measurement of JSRV Env fusogenicity

To better understand the JSRV Env fusogenicity and kinetics, we next developed a cell-cell fusion assay that could measure the redistributions of cytoplasmic contents once cells became fused. To facilitate this assay, we first used lentiviral vector transduction to establish a 293T effector cell line that stably expressed a green fluorescent protein (GFP). One clone that expressed an intermediate level of GFP (referred to as 293T/GFP) was chosen as the effector cell line for the cell-cell fusion assay described below.

293T/GFP cells were transfected with plasmids encoding individual Env; 24 h post-transfection, cells were co-cultured with target HTX/LH2SN cells pre-loaded with
CMTMR, an orange fluorescent dye that is activated by cellular esterases and retained in the cytoplasm. The conditions for the CMTMR-labeling were optimized so that the orange fluorescent cytoplasmic content signals in the target cells were comparable to that of GFP in the Env-expressing effector cells in order to facilitate FACS analysis. Co-cultured cells were treated with either neutral pH or pH 5.0 for 1 min, rapidly brought to neutral pH and further incubated at 37°C for 1 h, after which cell-cell fusion was analyzed by fluorescent microscopy and flow cytometry. Similar to syncytium formation (Fig. 1A), no obvious cell-cell fusion was observed at neutral pH for JSRV Env, nor for VSV-G and 10A1 Env (Fig 2A, left panels). Interestingly, FACS analysis reproducibly showed low levels of cell-cell fusion at neutral pH for VSV-G and JSRV Env (2 - 3-fold above the no-Env control) (Fig 2B). Co-culturing of effector and target cells at 4°C for 1 or 2 h did not reduce the low levels of fusion activities at neutral pH but rather yielded similar results (data not shown), indicating that the observed fusion at neutral pH was not due to co-culturing at 37°C.

Importantly, a pH 5.0 treatment markedly enhanced cell-cell fusion by both JSRV Env and VSV-G, but not by 10A1 MLV Env, as evidenced by the appearance of giant cells fluorescing both green and orange that appear yellow in the merged fluorescent images (Fig 2A, right panels) and by significantly increased percentages of GFP⁺/CMTMR⁺ double positive cells examined by FACS (Fig 2B, bottom panels). These results indicated that JSRV Env fusion is greatly enhanced by low pH.
**Cell-cell fusion induced by JSRV Env is Hyal2-specific**

Given the low yet detectable level of cell-cell fusion activity induced by JSRV Env at neutral pH, we next determined if the fusion by JSRV Env was Hyal2-specific. NIH 3T3 (TK⁻) cells that do not express a functional Hyal2 and NIH 3T3/LH2SN (TK⁻) cells that were engineered to express human Hyal2 [296] were used as target cells, respectively. At neutral pH, no apparent cell-cell fusion (< 1%) was observed for either cell line (Fig 3A). A brief exposure to pH 5 resulted in approximately 5% of NIH 3T3/LH2SN cells fusing, but did not give 3T3 cell fusion (Fig 3A). The relatively low percentages of fusion induced by JSRV Env in NIH 3T3/LH2SN cells was in a sharp contrast to that of VSV-G, which induced 20-25% cell-cell fusion with both NIH 3T3 and NIH 3T3/LH2SN cell lines (Fig. 3A).

We next performed the assay using HTX and HTX/LH2SN as target cells, in parallel with that of NIH 3T3 and NIH 3T3/LH2SN cells. To ensure comparability, exactly the same Env-transfected 293T/GFP dishes were split and used for co-culturing with these four different target cell lines. Cell-cell fusion was detected for JSRV Env in HTX/LH2SN cells, but not in HTX cells, at pH 5 (Fig. 3B), consistent with the results of syncytium formation assay (Fig. 1B). In contrast, VSV-G induced substantial and comparable levels of cell-cell fusion in both cell lines at low pH, but not at neutral pH (Fig. 3B, lower panels). These results for VSV-G were apparently different from what was observed in NIH 3T3 and NIH 3T3/LH2SN cells (Fig. 3A, upper panels), suggesting that the VSV-G induced fusion at neutral pH in NIH 3T3 cells was cell-type specific. Importantly, these data confirmed that an overexpression of Hyal2 was required for JSRV Env fusion in HTX cells.
Progressive truncation of JSRV Env CT renders syncytium formation at neutral pH that is further enhanced by a low pH treatment

We next investigated a possible role of the cytoplasmic tail of JSRV Env in fusion using the newly developed syncytium formation assay. A series of truncation mutants were created at the C-terminus of JSRV Env, namely CT608, CT599, CT589, CT574, CT572, and CT571 according to the position of the terminal amino acid (Fig. 4A). All mutants were tagged with a FLAG sequence at the N-terminus of SU [352] and their surface expression levels were determined by an anti-FLAG antibody using flow cytometry. As shown in Fig. 4B, all these mutants were efficiently expressed on the surface of 293/LH2SN cells, the cell line used for syncytium formation, and were roughly comparable among the mutants and wildtype.

The syncytium-forming activities of these truncation mutants were then assessed in 293/LH2SN cells. At neutral pH, CT608 and CT599 showed no apparent syncytium formation, similar to that of wildtype (Table 1). Intriguingly, mutants with further truncation towards the membrane spanning domain (MSD) of JSRV Env exhibited a progressively increased syncytium formation at neutral pH (Table 1). Yet the complete truncation of the CT in mutant CT571 resulted in a background level of fusion similar to wildtype (Table 1). Mutants CT574 and CT572, in particular, induced approximately five to eight-fold more syncytia (Table 1), of larger size, compared to that of wildtype Env (data not shown). A pH 5 treatment substantially enhanced the syncytium formation of all Env constructs, including CT571, whose fusion activity at low pH was apparently lower than that of wildtype Env and other truncation mutants (Table 1). Again, the two severe truncation mutants, CT574 and CT572 exhibited faster kinetics within the first 10 min of
pH 5 treatment, relative to the wildtype Env and other mutants (Table 1). Taken together, these results indicate that progressive truncation of JSRV Env renders syncytium formation at neutral pH that is further enhanced by a low pH treatment.

**Cell-cell fusion activities of JSRV Env truncation mutants**

The cell-cell fusion assay allowed us to quantitatively measure the fusion activities of JSRV Env truncation mutants. At neutral pH, low levels of cell-cell fusion activities were detected for all mutants, with no significant differences among these mutants and the wildtype observed (Fig. 5A). A pH 5 treatment resulted in substantially increased cell-cell fusion activities for all those mutants, including CT608 and CT571 whose fusion activities were low compared to other mutants (Fig. 5A). Under fluorescent microscopy, significant numbers of yellow GFP and CMTMR double positive cells were detected at pH 5 (data not shown). While it was difficult to accurately quantify fused cells using fluorescent microscopy, it was noticeable that the sizes of cell-cell fusion induced by the severe truncation mutants, in particular CT574 and CT572, were consistently greater than that of wildtype and CT571 (data not shown). These results were in agreement with that of syncytium formation assays (Table 1).

The surface expression of JSRV Env truncation mutants on 293T/GFP cells ranged from 50 to 75% of the level seen for wildtype Env as judged in terms of mean fluorescence intensity per cell (Fig. 5B). The low surface expression detected was particularly apparent for CT608, CT574, CT572, and CT571. To address this issue, we transfected 293T/GFP cells with different amounts of plasmid DNA encoding the JSRV Env wildtype or some of these mutants, ranging from 0.5 to 4 μg, and found that, the more the Env was expressed,
the higher the fusion activity was detected (Fig. 5C). Importantly, despite similarly low levels of surface expression, mutants CT574 exhibited an overall 2-3 fold higher fusion activity relative to that of wildtype Env, while CT608 fusion activity was similar to that of wildtype, and CT571 had an approximate two-fold reduced fusion activity compared to the wildtype (Fig. 5C). A graph of the relative surface expression versus relative fusion activity appeared to be linear, with squared correlation coefficient ($R^2$) up to greater than 0.9 for most of the constructs tested (Fig. 5C). The correlation clearly demonstrated that the fusion activity of JSRV Env was linearly dependent on the surface expression, and indicated that severe truncation of JSRV Env CT did enhance its cell-cell fusion activity at low pH.

**Fusion kinetics of JSRV Env at neutral pH is slow and is enhanced by CT truncations**

Noting that some of the JSRV Env CT truncations, in particular those proximal to the MSD, resulted in apparent syncytium formation at neutral pH (Table 1) but failed to show enhanced cytoplasmic content transfer relative to the wildtype (Fig. 5A), we investigated if this apparent discordance was due to experimental conditions used in our cell-cell fusion assays. The period of co-culture was expanded from the original 1 h period to 2, 5, 10, 20 and 30 h, respectively. While an extension of co-culture to 2- or 5 h had no significant effect on the fusion activities of constructs tested at neutral pH (Fig. 6A, and data not shown), prolonged co-culture revealed that after the initial few hours the truncation mutants CT589, CT574, and CT572 induced more rapid and extensive fusion that did not peak even by 30 h. This was in spite of the fact the surface expression levels of these constructs were 25-50% lower than that of wildtype (Fig. 5C). By contrast, fusion by VSV-G and wildtype JSRV Env peaked by 20 h at levels that were about half that of the two most active truncation mutants. These results were confirmed by fluorescence microscopy,
where a substantial proportion of GFP\(^+/\)CMTMR\(^+\) double-positive cells were observed, with sizes of fused cells increasing over time (data not shown). Collectively, these results demonstrate that progressive truncation of JSRV Env CT enhanced the cell-cell fusion activity of JSRV Env at neutral pH, although a prolonged co-culture was required.

**Kinetics of JSRV Env-mediated fusion at low pH is rapid**

The fusion kinetics of JSRV wildtype Env and representative truncation mutants were also determined at low pH. In this case, the effector and target cells were co-cultured at 37°C for 1h and were treated with pH 5 for 1 min as in the standard assay described above, and cell-cell fusion was analyzed following various periods of neutralization or recovery time, i.e., 5 min, 15 min, 30 min, 1 h and 2 h, respectively. Surprisingly, even within 5 min of pH 5-treatment, fusion reached peak levels, with only small changes afterwards for all constructs (Fig. 6B). These results suggested that fusion by JSRV Env and truncation mutants at pH 5 occurred rapidly and was almost complete within 5 min of pH 5 trigger.

We next shortened the recovery period to 0, 1, 3, and 4 min, respectively, and determined their effects on the fusion kinetics of JSRV Env and truncation mutants. Surprisingly again, a pH 5 treatment alone, even in the absence of a recovery time (time point 1 in Fig. 6C), resulted in an approximately 80% of the maximal fusion activities for all the JSRV Env tested, including CT571 which showed the lowest fusion activity at all time points examined (Fig. 6C). An additional one-minute recovery incubation at 37°C (time point 2 in Fig. 6C) substantially increased their fusion activities to peak levels, yet to comparable extents for all constructs, except for CT571 which exhibited a much smaller
increase compared to others (Fig. 6C). Of note, here also, despite their overall low levels of surface expression compared to that of wildtype Env, the fusion activity and kinetics of CT574 and CT572 were comparable to that of JSRV wildtype Env (Fig. 6C). These results, together with those shown in Fig. 6B, strongly suggested that cell-cell fusion induced by JSRV Env and truncation mutants at low pH occurred very rapidly, almost within the first 2 min after a pH 5 trigger. For comparison, fusion by VSV-G peaked at 1 min (~37% in fusion activity), followed by a decline at 2 min (~35%) and 4 min (~24%), respectively, and with a plateau at 5 min (23%) (data not shown).

**Bafilomycin A1 inhibits transduction by JSRV Env-coated pseudovirions**

The ability of JSRV Env truncation mutants to induce syncytium formation and enhance cell-cell fusion at neutral pH prompted us to examine if infection was affected by the endosomal proton pump inhibitor BafA1, an assay commonly used to determine the pH-dependence of virus entry. MoMLV pseudovirions bearing wildtype JSRV Env or individual truncation mutants were titered on 3T3/LH2SN cells by quantifying the GFP transducing units per ml (Fig 7A). The infectivity of truncation pseudotypes and the wildtype were comparable (Fig. 7A).

BafA1 strongly inhibited transduction by the wildtype JSRV pseudotypes (Fig. 7B), consistent with the pH-dependent fusion by JSRV Env observed above and with the studies we report in the accompanying article [353]. Interestingly, transduction by the CT574 and CT572 pseudotypes was also strongly, and almost equally, inhibited by the BafA1 treatments, either at 10 nM or 25 nM of BafA1 (Fig. 7B) and at different multiplicities of infections (MOIs, 0.1-0.5) (data not shown). Similar results were also obtained using
pseudovirions transducing alkaline phosphatase (data not shown). To explore if BafA1 might have any effect on the entry kinetics of JSRV pseudovirions bearing the wildtype or mutant Env, NIH 3T3/LH2SN cells were pre-bound by pseudovirions at 4°C for 2 h, followed by an incubation at 37°C for 4-6 h in the presence of 25 nM BafA1 added at 0, 1, 2 and 3 h following the temperature switch. Again, the extents of inhibition by BafA1 on the wildtype and these truncation mutants at every time point were similar (data not shown). Taken together, we concluded that while progressive truncation of JSRV Env enhanced cell-cell fusions at neutral pH, the cell-entry by pseudovirions bearing these truncations was still predominantly pH-dependent.

**Secretion of JSRV SU to culture medium is enhanced by CT truncations**

As an initial effort to understand possible mechanisms by which truncation of JSRV Env CT enhances its fusogenicity, we examined the synthesis, processing and release of SU into the culture medium in metabolic labeling experiments. 293T cells transiently transfected with plasmids encoding individual Env were pulse labeled with 35-S-Met/Cys for 1 h, then the label was chased for 4 h. Cell lysates and culture medium were separately subjected to immunoprecipitation with an anti-FLAG antibody that recognizes the FLAG sequence tagged at the N-terminus of SU, followed by SDS-PAGE and autoradiography. All the JSRV Env truncation mutants were efficiently synthesized and processed, with efficiencies approximately similar to the wildtype (Fig. 8A). Strikingly, release of SU to culture medium was markedly higher for CT574, CT572 and CT571, by almost 10-fold compared to that of wildtype and other mutants (Fig. 8B). Noticeably, the associations between the SU and TM subunits of these three truncation mutants decreased relative to that of wildtype and other mutants, although it was difficult to quantify these differences.
because of overall low intensities of TM (Fig. 8A). Nonetheless, these results demonstrated that secretion of JSRV SU was enhanced by the CT truncations, possibly resulting from conformational changes of the ectodomain of TM.

2.6 Discussion

We and others have previously attempted to investigate JSRV Env-mediated fusion with little success, largely because of insufficient levels of Hyal2 expression in the target cells and low transfection efficiency of effector cells used in these assays. In the present study, we overcame these problems by generating the 293/LH2SN and HTX/LH2SN cell lines that overexpress human Hyal2. Using these cell lines we developed an effective syncytium formation assay and a fluorescence-based cell-cell fusion assay that quantifies cytoplasmic content transfer and employed them to investigate the fusogenicity of JSRV Env. We demonstrated that rapid activation of JSRV Env fusion activity requires a low pH treatment and is Hyal2-specific (Fig. 1, 2 and 3; Table 1).

In this study we discovered that the CT of JSRV Env modulates its fusion activity and that the fusion-regulating region within its CT appears to differ in location from the fusion-modulating R peptide found in many other retroviral Env CTs [263, 264, 338-342]. Our results strongly argue that the N-terminal and central regions of CT (aa 572-599) of JSRV Env, rather than its C-terminus (600-615), are critical for fusion modulation. Truncation of the last sixteen aa of JSRV Env (CT599) had no significant effect on syncytium formation and cytoplasmic transfer activity (Table 1, Fig. 4 and 5). Further truncations to within one residue of the MSD, e.g., CT589, CT574 and CT572, progressively enhanced fusion (Table 1; Fig. 4, 5 and 6). In contrast, others have shown
that truncation of the CT of ecotropic Moloney MLV [263, 338, 354] and Mason-Pfizer monkey virus (M-PMV) [355, 356] to within one residue of the MSD does not increase fusion and fusion is not enhanced by progressively truncating its CT to lengths shorter than the natural R peptide cleavage site. We noticed also that the tailless CT571 exhibited a reduced fusion activity, which was possibly due to a disruption of Env membrane structure resulting from the loss of arginine (R572) residue adjacent to MSD. It has been previously shown that the positively charged amino acids located either in the membrane-proximal CT or within MSD of some enveloped viruses can influence the Env fusion activities [252, 357, 358].

More intriguingly, several JSRV Env CT truncations, in particular those proximal to the MSD (e.g., CT574 and CT572), induced syncytia (Table 1; Fig. 4C) and enhanced cytoplasmic transfer at neutral pH (Fig. 6A), although the fusion activity at neutral pH required a much longer incubation period than did fusion at acidic pH (Fig. 6B and C). Hence, progressive truncation of JSRV Env CT not only enhanced Env-mediated cell-cell fusion but also appeared to render it less dependent on low pH. This unexpected finding raised the possibility that low pH might be the trigger that relieves JSRV Env from the fusion modulation imposed by this CT region. That is, exposure to low pH might be providing the same function for JSRV Env as viral protease cleavage of the C-terminal R peptide does for many other retroviral Env.

This possibility predicted that the CT truncations should render virus infection less dependent on low pH. To test this concept, we examined the effect of the proton pump inhibitor BafA1 on infection of MoMLV pseudovirions bearing these truncated JSRV Env. Interestingly, these viruses were still as sensitive to BafA1 treatments as virus containing
wildtype Env (Fig. 7B). Two interpretations occur to us: the first is that the modulation of fusion by the CT is not relieved by acidic pH; the second is that low pH relieves the modulation of fusion by the CT but acidic pH is also required to trigger the initial conformation changes that expose the fusion peptide. We favor the latter case as the reason because this possibility seems most in line with the observation that low pH rapidly triggers syncytium formation and cell-cell fusion by JSRV Env (Table 1; Fig. 1, 2, 6B and 6C) and that the syncytium formation and cytoplasmic transfer induced by the JSRV truncation mutants at neutral pH were still strongly enhanced by a low pH treatment (Table 1 and Fig. 5).

One demonstrable effect of the CT truncations was altered conformations in the ectodomain of TM that may directly promote fusion. Release of SU from CT574, CT572 and CT571 into culture medium was markedly greater than that of wildtype Env and other mutants (Fig. 8B). Others have shown that deletion of the R peptide of MoMLV Env or truncation of HIV-1 or SIV Env CT induce conformational changes in the ectodomain of TM, and can result in increased SU shedding and cell fusion [269, 359, 360]. It is possible that the secreted SU may function as soluble receptor-binding domain (RBD) that promotes Env fusion at neutral pH, and in the case of JSRV, this may partially relieve the low pH requirement. However, conditioned medium of cells expressing truncation mutants that fuse at neutral pH did not increase in the fusion activity of wildtype JSRV Env at neutral pH, and a purified soluble SU did not render the JSRV entry less sensitive to BafA1 treatment (data not shown). Alternatively, the unconstrained TM on the surface of cells may directly enhance the Env fusion activity, as has been demonstrated for HIV-1 and other retroviruses [177]. Characterization of the conformational changes induced by the CT truncations
should provide important clues for a better understanding of the mechanisms of JSRV Env-mediated fusion and its regulation.

We propose two mechanisms that may explain an inhibitory effect of JSRV Env CT on fusion. First, similar to several other retroviruses [354, 356, 361], the N-terminus of JSRV Env CT preferentially folds into an α-helical structure [322] that facilitates coiled-coil formation (Y.-M. Zheng and S.-L. Liu, unpublished results) and as such may greatly stabilize the multimeric structure of the pre-fusion Env trimer. Whether or not the truncations presented here affect JSRV Env multimerization remains to be investigated. Second, any cellular factor(s) that binds to the Env CT to trigger intracellular signaling and thereby induce oncogenic transformation [325] may also be involved in regulation of fusion, directly or indirectly. In fact, the severely truncated JSRV Env mutants that exhibited enhanced fusogenicities, i.e., CT589, CT574 and CT572, were incapable of inducing cell transformation in vitro (Y.-M. Zheng and S.-L. Liu, unpublished results). Alternatively, an independent cellular factor(s) unrelated to transformation might be responsible for fusion regulation by the Env CT. Work is ongoing to identify cellular binding partners of JSRV Env CT, and these molecules, once identified, will be tested for their roles in cell fusion.

The betaretrovirus family to which JSRV belongs differs in their pH-dependence and in the presence or absence of a cleavable R peptide in the CT of their TM. MMTV entry is pH-dependent and the CT of its TM is not cleaved by the viral protease [356], whereas, MPMV entry is pH-independent and its Env has a C-terminal 16 residue R peptide that is cleaved off by the viral protease after budding [341, 355]. While our current data showing the pH-dependence of JSRV Env-mediated fusion and its negative regulation
by the CT does not implicate for a role or a presence of R peptide in the JSRV Env CT, they do not rule out this possibility either and it will have to be critically addressed in the future studies. One essential question that remains to be addressed is the evolutional and functional role of JSRV Env CT in vivo, particularly from the viral replication and oncogenesis perspectives. In this respect it is of interest to note that the pH of human and likely other mammal’s lung fluids are relatively acidic [362], and that JSRV MoMLV pseudovirions are quite resistance to the denaturing environment of lung fluids [363] and to low-pH inactivation [364]. Whether or not the pH-dependent entry of JSRV reported here is also important for viral replication in vivo remains to be investigated.

2.7 Acknowledgements

We thank A.D. Miller for reagents, and for continuous support and encouragements. We also thank Eric Cohen for helpful discussions. This work was supported by the Canadian Institute of Human Research (CIHR) to S.-L.L, and a grant from the US National Institutes of Health to L.M.A (AI 33410). M. C. was supported by scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Fonds de la Recherche en Santé du Québec (FRSQ). S.-L. Liu is a Canada Research Chair in Virology and Gene Therapy.
Table 2.1 Syncytium induction by JSRV Env and truncation mutants

<table>
<thead>
<tr>
<th>Env</th>
<th>Syncytium formation (per field)</th>
<th>pH 7.4</th>
<th>pH 5.0</th>
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<tr>
<td></td>
<td></td>
<td>10 min</td>
<td>15 min</td>
</tr>
<tr>
<td>None</td>
<td>0.5 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WT</td>
<td>1.5 ± 0.6</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>CT608</td>
<td>1.3 ± 0.5</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>CT599</td>
<td>2.8 ± 1.2</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>CT589</td>
<td>7.3 ± 1.0</td>
<td>+++</td>
<td>+++++++</td>
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<tr>
<td>CT574</td>
<td>11.5 ± 1.9</td>
<td>+++++</td>
<td>+++++++</td>
</tr>
<tr>
<td>CT572</td>
<td>13.8 ± 3.1</td>
<td>+++++</td>
<td>+++++++</td>
</tr>
<tr>
<td>CT571</td>
<td>1.5 ± 0.6</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>VSV-G</td>
<td>3.0 ± 1.1</td>
<td>++++++</td>
<td>+++++++</td>
</tr>
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</table>

293/LH2SN cells transfected with plasmids encoding JSRV Env WT, truncation mutants, or VSV-G were evaluated for syncytium induction at pH 7.4 and 5, respectively. Transfection efficiencies were similar for all constructs as determined by the GFP signals encoded by a co-transfected plasmid.

*a*: numbers of syncytia per field at pH 7.4; averaged results from at least four fields; syncytia with 4 nuclei or greater were counted.

*b*: percentages of syncytium formation per field 10 or 15 min after a pH 5.0 treatment. +: 10-20 %; ++: 20-40 %; +++: 40-60%; ++++: 60-90 %; ++++++: >90 %. Results are from a representative experiment. Same experiments were repeated at least 4 times, with similar results.
Fig. 2.1 JSRV Env protein induces syncytium formation at low pH in cells overexpressing human Hyal2. 293/LH2SN (A) or HTX/LH2SN (B) cells were transfected with plasmid DNA encoding JSRV Env, VSV-G, 10A1 MLV Env, or none (No Env); 12-24 h post-transfection, cells were examined for syncytium induction at neutral pH and phase contrast micrographs of representative fields captured (pH 7.4, top panels). Cells were then treated with a pH 5.0 buffer for 1 min, after which the pH was brought back to neutral and cells were examined for syncytium induction under a light microscope over a period of 5 min to 24 h after the low pH pulse. Images shown are representative phase contrast micrographs captured at the 1 h time point (pH 5.0, bottom panels). Arrows indicate syncytia induced by JSRV Env in HTX/LH2SN cells. (C) Hyal2 surface expressions in cells used in syncytium induction assays. 293 (solid line), 293/LH2SN (broken line), HTX (solid line) and HTX/LH2SN cells (broken line) were incubated with purified JSRV SU-human IgG Fc fusion protein, followed by an incubation with FITC-labeled anti-human Fc antibody, and were analyzed by flow cytometry. Filled areas represent 293/LH2SN and HTX/LH2SN cells incubated with secondary antibody alone. Representative results of three independent experiments are shown.
Fig. 2.2 JSRV Env protein induces cell-cell fusion at low pH. Effector 293T/GFP (green) cells were transfected with plasmid DNA encoding JSRV Env, VSV-G, or none (No Env); 16-24 h post-transfection, cells were counted and co-cultured at 37°C for 1 hour with HTX/LH2SN target cells prelabeled with CMTMR (red). Cells were then treated with pH 7.4 or pH 5.0 buffers for 1 min, cultured in regular medium for an additional 1 h, and cell-cell fusion was analyzed by fluorescent microscopy (A) or flow cytometry (B). Arrows indicate fused cells (orange-yellow in the overlay images) induced by JSRV Env or VSV-G. Numbers shown in the upper right quadrant of cytometry profiles represent percentages of fused cells.
Fig. 2.3 Cell-cell fusion induced by JSRV Env protein is receptor-dependent and requires an overexpression of Hyal2. Cell-cell fusion was performed as described in Fig. 2, except that NIH 3T3 and NIH 3T3/LH2SN (A), or HTX and HTX/LH2SN (B) cells were used as target cells, respectively. Percentages of CMTMR-GFP double positive cells, indicative of fusion, were determined by flow cytometry using the FlowJo program. Values are the means of a representative experiments performed in duplicate. These experiments were repeated three or four times, with similar results.
Fig. 2.4. The Cytoplasmic tail (CT) sequences and surface expressions of JSRV Env wildtype and truncation mutants. (A) The CT sequences of JSRV Env wildtype (WT) and truncation mutants. Amino acid numbering of JSRV Env WT was according to JS7 strain [348], and truncation mutants were named according to the positions of their terminal amino acids. MSD: membrane-spanning domain. Note that all constructs were tagged with a FLAG tag sequence at the N-terminus of SU. (B) Env surface expression in 293/LH2SN cells. Cells transfected with plasmids encoding JSRV Env WT or truncation mutants were incubated with an anti-FLAG antibody, and examined for Env expression by flow cytometry. The fluorescence intensities (geometric means) of truncation mutants were normalized relative to that of WT (%). Values are the means of four independent experiments ± standard deviations.
**Fig. 2.5 Cell-cell fusion activities of JSRV Env CT truncation mutants.** Cell-cell fusion assays were performed as described in Fig. 2, except that different JSRV Env CT truncation mutants were used for transfection of 293T/GFP cells. (A) Relative fusion efficiency measured by flow cytometry. Cell-cell fusion was performed the same as described in Fig. 2. The fusion activities of CT truncation mutants were compared to that of wildtype (WT) Env at pH 5 (which was set to 100%). Results are averages of three independent experiments performed in duplicate ± standard deviations. An asterisk represents $p < 0.05$, indicating that the difference between the truncation of interest and the WT was statistically significant. (B) Relative Env surface expression of JSRV Env CT truncation mutants in 293T/GFP cells. A portion of transfected 293T/GFP cells were incubated with an anti-FLAG antibody to determine the Env surface expression by flow cytometry. The average fluorescence intensities per cell (geometric means) of truncation mutants were normalized to that of WT (%); values are averages of four independent experiments ± standard deviations. An asterisk indicates $p < 0.05$. (C) Correlations between Env surface expression and cell-cell fusion activity at low pH. 293T/GFP cells were transfected with varying amounts of plasmid DNA encoding JSRV Env WT, CT608, CT574 or CT571, ranging from 0.5 to 4 μg. Env surface expression and cell-cell fusion activity at pH 5 were determined by flow cytometry. For analysis, the highest surface expression of JSRV Env WT and its corresponding fusion activity were set to 100% in x-axis and y-axis, respectively, and were used to normalize the other data. Values are averages of two independent experiments ± standard deviations; $R^2$ was calculated from each experiment and was averaged.
A

Relative fusion (%)

- pH 7.0
- pH 5.0

WT CT608 CT599 CT589 CT574 CT572 CT571 No Env

B

Relative expression (%)

WT CT608 CT599 CT589 CT574 CT572 CT571

C

Relative fusion (%)

pH 5.0

CT574 (R² = 0.92)

WT (R² = 0.93)

CT608 (R² = 0.92)

CT571 (R² = 0.75)
Fig. 2.6 Fusion kinetics of JSRV Env and truncation mutants. Cell-cell fusion assays were performed as described in Fig. 2, except that different JSRV Env truncation mutants were used for transfection of 293T/GFP cells and that different periods of recovery time were applied to co-cultured cells after a pH 7.4 or pH 5 pulse. (A) Fusion kinetics at neutral pH. Following 1-min treatment with a pH 7.4 solution, co-cultured cells were incubated at 37°C for 2, 5, 10, 20 and 30 h, respectively, and cell-cell fusion activity was measured for each construct. (B) and (C). Fusion kinetics at low pH. Following 1-min treatment with a pH 5 solution, co-cultured cells were incubated with regular medium at 37°C for the indicated time periods, and fusion activity was determined by flow cytometry. Results are from a representative experiment performed in duplicate; experiments were repeated at least three times, with similar results. Note that the Env surface expressions of CT574, CT572 and CT571 in these experiments were approximately 25-50% lower than that of WT (similar to the results shown in Fig. 5B), and fusion activities presented here were not normalized by their expression levels.
Fig. 2.7 MoMLV pseudovirions bearing JSRV Env wildtype (WT) and CT truncations are sensitive to Bafilomicin A1 (BafA1) treatment. (A) NIH 3T3/LH2SN cells were infected with GFP-encoding MoMLV pseudovirions bearing JSRV Env WT or truncation mutants, and percentages of GFP⁺ cells were determined by flow cytometry 48 h post-infection. Titers are expressed as GFP transducing units (TU) per ml. (B) NIH 3T3/LH2SN cells were pretreated with indicated concentrations of BafA1 for 2 h, and infected by MoMLV pseudovirions bearing different Env (MOI = 0.1-0.5) in the presence of the drug for 6 hours. Noninternalized viral particles were inactivated by citrate buffer at pH 3.15 for 1 min, and percentages of GFP positive cells were determined 48 h post-infection by flow cytometry. Values are percentages of transduction (%) relative to untreated cells and are the averages of two independent experiments ± standard deviations.
Fig. 2.8. Severe truncation of JSRV Env CT results in increased release of SU into the culture medium. 293T cells transfected with plasmid DNA encoding JSRV Env wildtype (WT) or CT truncation mutants were pulse labeled with $^{35}$S-Met/Cys for 1 h, and chased for 4 h. Cell lysates and culture medium were harvested and immunoprecipitated using anti-FLAG beads. The immunoprecipitated products were resolved by SDS-PAGE, and analyzed by autoradiography. (A) Synthesis and processing of JSRV Env proteins in cell lysates. Positions of radiolabeled JSRV Env precursor (Env), SU, and TM that were recovered in the immune complexes precipitated by antibody against the FLAG tag on SU are indicated by arrows. An unrelated JSRV Env mutant having a FLAG sequence at both N-terminus of SU and the C-terminus of TM (first lane, F-Jenv-F) was also included in these experiments in order to indicate the positions of TM. (B) Release of JSRV SU in wildtype and truncation mutants into cell culture medium. The radiolabeled SU recovered in the immune complexes from each culture medium sample was quantified from densitometric scans of the autoradiographs using Quantity One software. The band intensity of SU recovered from the WT sample was set to 1, and the relative intensity of each CT mutant was calculated as the ratio of the intensity of the mutant SU band to that of the WT sample. Results shown are the averages of two independent experiments. No Env: samples from parental, non-transfected 293T cells.
CHAPTER 3

ENZOOTIC NASAL TUMOR VIRUS ENVELOPE

REQUIRES A VERY ACIDIC pH FOR

FUSION ACTIVATION AND INFECTION

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3.1 Preface to Chapter 3

The previous study presented in Chapter 2 revealed that a low pH trigger is critical for the activation of JSRV Env-induced fusion. Because the Env of JSRV and ENTV share a high sequence identity, we sought to know if ENTV also requires a low pH for entry and fusion. In addition, since both viruses use Hyal2 as their cell entry receptor, it was possible to use our newly developed cell-based fusion assay for the study of ENTV Env fusion activation. However, the affinity of ENTV for human Hyal2 was shown to be much lower than that of JSRV, which results in a low titer of pseudovirions harbouring ENTV Env compared to those that have JSRV Env at their surface [283, 302].

Interestingly, experiments revealed that, unlike JSRV Env, ENTV Env was unable to mediate cell fusion following a 1min pH 5.0 treatment. Surprisingly, ENTV Env required a more acidic pH for fusion and its fusion activity was lower than that of JSRV Env. Given that the data presented in the previous chapter highlight the importance of receptor overexpression, we wanted to explore the possibility that ENTV’s low fusion activity is due to the inherently low Hyal2 binding affinity of its SU. Towards that end, we generated chimeras of JSRV and ENTV SU and TM. The data presented in this chapter underlines the differences between JSRV and ENTV Env-mediated fusion and their consequences on viral infection.
3.2 Abstract

Enzootic nasal tumor virus (ENTV) is a close relative of Jaagsiekte sheep retrovirus (JSRV), and both viruses use the same receptor, hyaluronidase 2 (Hyal2), for cell entry. We report here that, unlike JSRV envelope (Env), the ENTV Env protein does not induce cell fusion at pH 5.0 and above, but requires a much lower pH (4.0-4.5) for fusion to occur. Entry of ENTV Env pseudovirions was substantially inhibited by bafilomycin A1 (BafA1), but was surprisingly enhanced by lysosomotropic agents and lysosomal protease inhibitors following a 4-6 h treatment period; of note, a prolonged treatment with BafA1 or ammonium chloride completely blocked the ENTV entry. Unlike typical pH-dependent viruses, ENTV Env pseudovirions were virtually resistant to inactivation at low pH (4.5 or 5.0). Using chimeras between ENTV and JSRV Env, we demonstrated that the transmembrane (TM) subunit of ENTV Env is primarily responsible for its unusually low pH requirement for fusion, but found that the surface (SU) of ENTV Env also critically influences its relatively low and pH-dependent fusion activity. Furthermore, the poor infectivity of ENTV pseudovirions in human cells was significantly rescued by either substituting the SU subunit of ENTV Env with that of JSRV Env, or by over-expressing the functional Hyal2 receptor in target cells, suggesting that ENTV SU-Hyal2 interaction is likely the limiting step for viral infectivity. Collectively, our data reveal that the fusogenicity of ENTV Env is intrinsically lower than that of JSRV Env, and that ENTV requires a more acidic pH for fusion which may occur in an intracellular compartment(s) distinct from that of JSRV.
3.3 Introduction

Enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV) are two related simple betaretroviruses that cause contagious respiratory tumors in sheep and goats. ENTV targets the upper airway epithelial cells and induces nasal adenocarcinomas [365], whereas JSRV infects the lung airway epithelial cells causing pulmonary adenocarcinomas [366]. One unique feature of these sheep retroviruses is that their envelope (Env) function as active oncogenes inducing transformation in cultured cells [367-373] and causing tumors in animals [374-376]. Prior efforts have been devoted largely to an understanding of the mechanisms of oncogenic transformation by these Env proteins, in particular of JSRV, while fusion and cell entry mediated by these Env proteins are poorly understood. Recently, we showed that JSRV Env-mediated fusion requires a low pH, and that the cytoplasmic tail (CT) of JSRV Env negatively regulates its fusion activity [377, 378]. The mechanisms of ENTV Env-mediated fusion are currently not known.

As in all other retroviruses, ENTV Env is a type I transmembrane protein composed of surface (SU) and transmembrane (TM) subunits [379]. The SU subunit contains a receptor-binding domain that recognizes the entry receptor, hyaluronidase 2 (Hyal2). Hyal2 is a glycosylphosphatidylinositol (GPI)-anchored cell surface molecule that also serves as the receptor for JSRV [369, 371, 380]. The TM subunit of ENTV Env is directly involved in membrane fusion, and it contains an amino (N)-terminal fusion peptide, a central coiled-coil region, a membrane-spanning domain (MSD), and a carboxyl (C)-terminal CT [379]. One interesting feature of ENTV Env is that, similar to JSRV Env, it has a relatively long CT of 47 amino-acid residues [379]. More importantly, the CT of ENTV Env shares only ~50% sequence identity with that of JSRV at the amino acid level, in contrast to an overall
sequence identity of 89% for the full length Env [379]. The significance of these sequence differences between ENTV and JSRV Env is not known, but might be related to oncogenic transformation potential, Env trafficking, fusion modulation, or virus assembly [381].

ENTV uses human, ovine, or bovine Hyal2 for binding and entry in a fashion similar to JSRV, but appears to have a restricted host range [369]. A Moloney murine leukemia virus (MoMLV) oncoretroviral vector bearing JSRV Env efficiently transduced cell lines derived from most species, including sheep, human, bovine, and canine, with the notable exception of rodent cell lines [371, 382]. In contrast, an equivalent MoMLV vector bearing ENTV Env did not, or only weakly, transduced these cell lines, including those of humans and sheep [367, 369, 383]. Interestingly, over-expression of human Hyal2 rendered most of these cell lines highly transducible for ENTV pseudotypes, indicating that Hyal2 over-expression can overcome the inefficient binding of ENTV Env to Hyal2 [383]. Using soluble SUs of ENTV and JSRV Env that are respectively fused to a human IgG Fc fragment, it was shown that the binding affinity of ENTV Env for human Hyal2 is much lower than that of JSRV Env, with a dissociation constant (Kd) of ~175 nM and ~10 nM for ENTV and JSRV, respectively [352, 383]. A subsequent surface plasmon resonance study revealed an even tighter association between JSRV Env and human Hyal2, with a Kd of 32 pM [384], although similar study has not been performed for ENTV Env. Whether or not Hyal2 also plays a direct role in activation of ENTV and JSRV Env-mediated fusion is currently not known.

Using syncytium induction and cell-cell fusion assays that we recently developed for JSRV Env [378], herein we have examined the fusion properties of ENTV Env. We found, unexpectedly, that the fusogenicity of ENTV Env is much lower than that of JSRV Env and it requires a very acidic pH for fusion activation and cell entry. We determined
that the TM subunit of ENTV Env dictates the extremely low pH requirement for fusion, yet discovered that the SU subunit of ENTV Env also critically influences its relatively low and pH-dependent fusion activity, suggesting that Hyal2 may play a direct role in fusion activation. The remarkable differences in fusogenicity and low pH requirement between ENTV and JSRV Env proteins, as well as their responses to treatments by lysosomal protease inhibitors and lysosomotropic agents, strongly argue that ENTV may use an intracellular compartment(s) that is distinct from that of JSRV for its pH-dependent fusion and cell entry.

3.4 Materials and methods

Reagents and antibodies. The fluorescent dye 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR) and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Ammonium chloride (NH₄Cl) and chloroquine were purchased from Sigma (St. Louis, MO). Bafilomicin A1 (BafA1), leupeptin hemisulfate, cathepsin inhibitor III were purchased from Calbiochem (Darmstadt, Germany). The anti-FLAG monoclonal antibody beads (EZviewTM Red), anti-FLAG affinity gel, anti-β-actin monoclonal antibody, and the secondary anti-mouse IgG coupled to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were purchased from Sigma (St. Louis, MO). The rabbit anti-MLV Gag antiserum was a kind gift of Alan Rein (National Cancer Institute, Frederick, MD).

Env constructs and cell lines. The parental JSRV Env derived from the JS7 strain [385] and the parental ENTV Env derived from the ENTV1 strain [379] were cloned into the
pCI-Neo vector, with introduction of a FLAG sequence at both the N- and C-termini, as described previously [378, 386]. The chimeric Env between ENTV and JSRV was generated by swapping the SU and TM of each Env, resulting in EJ, which contains the ENTV SU and the JSRV TM, and JE, which contains the JSRV SU and the ENTV TM, respectively. The 10A1 amphotropic MLV Env-expressing vector and VSV-G-expressing plasmid have been described previously [378, 387]. The Ebola GP expression vector was a kind gift of Gary Kobinger (National Microbiology Laboratory, Winnipeg, Canada).

293T/GFP, 293/LH2SN, NIH 3T3/LH2SN, HTX/LH2SN, and 293/GP-LAPSN cells have been described previously [371, 378, 388]. All cell lines were cultured in Dulbecco’s Modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and were maintained at 37°C in a 10% CO2–air atmosphere at 100% relative humidity.

**Syncytium induction and cell-cell fusion assays.** The syncytium induction assay was performed as described previously [378]. Briefly, 1.7 × 10⁶ 293/LH2SN cells plated in 6-well plates were transfected with 2 μg of plasmid DNA encoding Env or VSV-G, plus 0.5 μg of pCMV-GFP-encoding plasmid (a kind gift from François-Loïc Cosset, Lyon, France), using the calcium phosphate method. At 24 h post-transfection, cells were treated with PBS-10 mM HEPES-10mM MES (Sigma) pH ranging from 3.0 to 7.0 for 1 or 5 min at 37°C. Cells were then incubated at 37°C in complete growth medium, and syncytium induction was examined 10 min to 24 h after treatment. Pictures were taken 1 h after treatment using a fluorescence microscope (Carl Zeiss, Goettingen, Germany) under a green filter for GFP expression.
The cell-cell fusion assay was also performed as previously described [378]. Briefly, $1.7 \times 10^6$ 293T/GFP cells were transfected with 2 µg of DNA encoding Env or VSV-G using the Lipofectamine 2000 method. At 24 h post-transfection, cells were detached using PBS plus 0.5 mM EDTA, and co-cultured at a 2:1 ratio with effector HTX/LH2SN cells that were pre-labeled with CMTMR as previously described [378]. After 1 h co-culture at 37°C, cells were treated for 1 min with PBS-10 mM HEPES -10 mM MES pH 4.0, 5.0 or 7.0, and allowed to recover in normal growth medium for 1 h. The cells were trypsinized and analyzed by flow cytometry using a FACScalibur (BD Bioscience, Mississauga, ON, Canada). The surface expression of Env was determined by anti-FLAG staining, and analyzed by flow cytometry as previously described [378].

**Pseudotyping, infection, and pH inactivation of pseudovirions.** GFP-encoding MoMLV pseudovirions were produced by co-transfecting $2.5 \times 10^6$ 293T cells with a plasmid encoding individual Env, VSV-G, or Ebola GP, a packaging plasmid encoding the MoMLV Gag-Pol proteins (pCMV-gag-pol-MLV) and a transfer vector encoding GFP (pCMV-GFP-MLV) (both plasmids were kind gifts from François-Loïc Cosset, Lyon, France). Alternatively, the packaging cell line 293/GP-LAPSN was transfected with a plasmid encoding each Env to produce alkaline phosphatase (AP)-expressing pseudovirions. Supernatants were harvested 48 to 72 h post-transfection, and cell debris was removed by centrifugation at 3,200 × g. Viruses were used immediately for infection or were stored at -80°C.

Target cells were infected with appropriate amounts of viral supernatant in the presence of 5 µg/ml polybrene (Sigma), and were assessed for GFP expression by flow
cytometry 48 h post-infection [378], or for AP-positive foci by cell staining 72 h post-infection as previously described [389]. For experiments involving inhibitors, cells were pre-treated with appropriate concentrations of agents for 1-2 h, followed by infection with viruses in the presence of drugs for 4-6 h or 16-48 h, unless otherwise stated. Comparable MOIs were used for all viral pseudotypes in each experiment, and typically an MOI of 0.05 to 0.2 was used for all infections. Non-internalized viruses were inactivated with citrate buffer (pH 3.15) after infection period, and viral infectivity was determined 48 h post infection. To assess the stability of ENTV pseudoparticles at different pH, GFP-encoding viruses were incubated in buffers ranging from pH 3.0 to 7.0 at 37°C for 30 or 5 min, followed by neutralization with culture medium. Appropriate amounts of viruses were used to infect HTX/LH2SN cells, and titers were determined 48 h by flow cytometry post-infection.

**Rescue of BafA1-mediated entry block by low pH pulse.** HTX/LH2SN cells were pre-treated with 30 nM BafA1 for 1 h, and incubated with ENTV Env pseudovirions at 4°C for 2 h. Following three washes with cold PBS, the virion-cell complexes were either directly exposed to a low pH solution ranging from 3.5 to 7.0 for 5 min, or were pre-incubated at 37°C for 1 h in the presence of 30 nM of BafA1, followed by incubation in a low pH solution for 5 min. In both cases, the total infection period was 4 h in the presence of 30 nM BafA1. Non-internalized virus was inactivated using citrate buffer (pH 3.15) after the infection period, and viral infectivity was determined by flow cytometry 48 h after the initiation of infection.
Cell cycle analysis. Cell cycle analysis was performed as previously described [390] in order to determine potential side-effects of BafA1 and lysosomotropic agents on cell proliferation. Briefly, HTX/LH2SN cells were treated with the highest concentrations of BafA1 or lysosomotropic agents, i.e., 30 nM BafA1, 30 μM chloroquine or 30 mM NH₄Cl, for 4 to 6 h, the same as that were used in the infection assays. Sixteen hours later, cells were trypsinized and fixed in ice-cold ethanol overnight. Cells were washed, and treated with 5 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO) and 100 μg/ml RNaseA (Qiagen, Chatsworth, CA) for 30 min at 37 °C, and were analyzed by flow cytometry using FACSscalibur (BD Biosciences) and FlowJo software (FlowJo, LLC., Ashland, OR).

Env incorporation into a MoMLV vector and immunoblotting. Virion-producing 293/GP-LAPSN cells were lysed in a lysis buffer as previously described [386], and pre-cleared by centrifuging for 10 min at 13,000 × g and 4 °C, followed by boiling for 5 min in sodium dodecyl sulfate (SDS)-loading buffer (0.4% SDS and 0.01% beta-mercaptoethanol, β-ME). Supernatant containing viral particles from the transfected 293/GP-LAPSN cells was first pre-cleared by centrifugation for 5 min at 3,200 × g and 4 °C. Viruses in the supernatant were purified by centrifugation on a 20% sucrose cushion for 3 h at 185,000 × g and 4 °C, and the pellet was resuspended in SDS-loading buffer (1% SDS and 0.02% β-ME) followed by boiling for 10 min. Cell lysates and viral particles were subjected to 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membranes and immunoblotting using an anti-FLAG antibody as previously described [386].
**Statistical analysis.** Statistical analysis was performed using two-tail student’s t test and was adjusted by Bonferroni corrections unless otherwise noted. Data plotted in all histograms or shown in the tables were means ± standard deviations, unless only two data points were included, for which only the means were given. Typically two to eight independent experiments were used for statistical analysis.

### 3.5 Results

**ENTV Env induces syncytium formation and cytoplasmic content transfer at pH 4.5 or below, but not at pH 5.0 or above**

We recently showed that the JSRV Env protein induces cell-cell fusion and syncytia formation following a 1 min treatment at pH 5.0 [378]. Given the high sequence similarity between ENTV and JSRV Env [369, 379, 385] and that both viruses use the same receptor for entry [369, 371], we asked whether ENTV Env also induces fusion under similar conditions. Unexpectedly, while JSRV Env and VSV-G induced extensive syncytia after a treatment at pH 5.0 for 1 min as previously shown [378], no syncytia were observed for ENTV Env (Fig. 1A, upper panel). In contrast, treatment of ENTV Env-expressing cells at pH 4.0 for 1 min induced substantial syncytia (Fig. 1A, upper panel), although it took longer for the syncytia to appear (~20 min) than it did for JSRV Env even at pH 5.0 (~10 min) [378]. Occasionally, a few syncytia were observed in cells expressing ENTV Env at pH 4.5, but the sizes of the syncytia were extremely small (Fig. 1A, upper panel). Noticeably, prolonged treatment of cells expressing ENTV Env for 5 min resulted in extensive syncytia at pH 4.5, and occasionally at pH 5.0, yet with very small sizes which were often indistinguishable from normal cells (Fig. 1A, lower panel). Taken together,
these results indicated that the fusogenicity of ENTV Env is much lower than that of JSRV Env, and that a more acidic pH (~pH 4.5) is required for the ENTV Env-mediated cell fusion to occur.

To confirm the above results and quantify the fusion activity of ENTV Env, we next used a cell-cell fusion assay that we recently developed for JSRV Env [378]. The fusion indexes for Env or VSV-G, as determined by the percentages of fusion at a certain pH relative to that at the optimal pH, were plotted against the pH values used (Fig. 1B). VSV-G exhibited close-to-maximal fusion activity over a broad pH range, with a sharp drop at ~pH 6.0, and fusion was undetectable at pH 6.5 (Fig. 1B). This pattern was similar to that reported previously [391, 392]. JSRV Env also exhibited fusion activity over a broad pH range, yet interestingly with maximal fusion at pH 3.5; its fusion activity was detected until pH 6.5, after which background fusion was observed as we previously shown [378] (Fig. 1B). In sharp contrast, ENTV Env-induced fusion occurred within a very narrow pH range; it started to appear at pH 4.0, and exhibited maximal fusion at ~pH 3.0, beyond which cells were essentially detached by lower pH treatments (Fig. 1B, and data not shown). Notably, the fusion activity of ENTV Env was also lower than that of JSRV Env, with approximately 15% that of JSRV Env at pH 4.0 (see details in Table 1 and Fig. 6B). The pH values corresponding to the half-maximal fusion activities of ENTV Env, JSRV Env and VSV-G were estimated to be 3.7, 5.4 and 6.2, respectively. It should be emphasized, however, that the cell-cell fusion assays shown here, and throughout this study, were performed with 1 min treatment of co-cultured cells, and because of technical limitations we were unable to treat the co-cultured cells for 5 min (cells became detached), as we did for the syncytium induction assay (Fig. 1A), in order to determine if ENTV Env can also cause cell-cell
fusion at pH 4.5. Nonetheless, these results confirm that ENTV Env indeed has a low fusogenicity and requires an unusually low-pH for fusion compared with that of JSRV Env.

The surface expression of ENTV Env SU, along with that of JSRV, was examined by flow cytometry in both 293/LH2SN and 293T/GFP cells. We consistently observed higher SU expression for ENTV Env than for JSRV Env in both cell lines (based on geometric means, Fig. 1C), indicating that the relatively low fusion activity of ENTV Env and its unusually low pH requirement are not due to the levels of ENTV SU expression on the cell surface.

**Entry of ENTV Env pseudovirions is pH-dependent, but the pattern appears to be different from that of JSRV**

The low pH-dependent fusion of ENTV Env predicted that ENTV entry should be pH-dependent. To test this, we generated MoMLV pseudovirions bearing the Env of ENTV, JSRV or 10A1, or VSV-G, and tested their ability to enter cells in the presence of bafilomycin A1 (BafA1), a specific, non-reversible endosomal proton pump inhibitor [393], as well as two commonly used lysosomotropic agents [394], ammonium chloride (NH₄Cl) and chloroquine. Due to the limited host range of ENTV [369], we used cell lines that over-express human Hyal2 in this and all other experiments throughout this study, unless otherwise specified. BafA1 inhibited ENTV Env pseudovirion infection in HTX/LH2SN cells by ~25% (p < 0.05 at 30 nM, n = 3), in contrast to that of JSRV Env and VSV-G which were inhibited by ~50-70% (p < 0.05, n = 4) and ~60-85% (p < 0.05 or 0.01, n = 3), respectively (Fig. 2A). As expected, entry by 10A1 Env pseudovirions was not affected by the BafA1 treatment (Fig. 2A), consistent with the fact that 10A1 is a pH-independent retrovirus [395, 396]. No apparent cytotoxicity was observed in the BafA1 treated cells,
which was also confirmed by independent cell cycle analyses showing similar cell proliferation profiles (G₀/G₁, M, G₂/M) to that of untreated cells (data not shown). Similar results were also obtained in human 293 and mouse NIH 3T3 cells over-expressing human Hyal2 (data not shown).

Treatment of cells with chloroquine or NH₄Cl yielded somewhat surprising results. While chloroquine strongly inhibited the entry of VSV-G pseudovirions by ~60-90% (p < 0.05, n = 4), its inhibitory effect on JSRV Env pseudovirions was much less, approximately 20-40% (p < 0.05, n = 4) following a similar 4 h treatment (Fig. 2B), as we previously observed [377]. Surprisingly, entry of ENTV Env pseudovirions was not inhibited, but was rather enhanced by chloroquine treatment, although the difference was not statistically significant (p = ~0.34, n = 4) (Fig. 2B). Even more strikingly, a 4-h NH₄Cl treatment performed in parallel significantly increased, in a concentration-dependent manner, the entry of ENTV Env pseudovirions (p < 0.05 and p < 0.01 for 15 and 30 mM, respectively, n = 3, Fig. 2C), while substantially blocking the entry of JSRV Env (~50%, p = 0.06, n = 3) and VSV-G (~80%) pseudovirions (Fig. 2C), similar to previous results [377]. Cell cycle analyses were also performed on HTX/LH2SN cells that were treated with 30 mM NH₄Cl or 30 μM chloroquine for 4-6 h, and no difference in cell cycle profiles was found between agent-treated and untreated cells (data not shown). The strong enhancement of ENTV entry by lysosomotropic agents might be due to inhibitory effects of these agents on viral particle degradation in the lysosomes (see related sections below), as has been shown for HIV-1 [397, 398], and other factors described below.

The relatively mild inhibitory effect of BafA1 and the apparent enhancement of lysosomotropic agents on ENTV entry also raised the possibility that ENTV Env pseudovirions that accumulated in the intracellular vesicles during the agent treatment
period may remain transduction competent, and once these agents are withdrawn from the cells, viral transductions could quickly resume as efficiently as that in the absence of agent treatment. To test this possibility, we treated HTX/LH2SN cells with BafA1, chloroquine, or NH₄Cl in the presence of viruses for a prolonged period of time, i.e., for 16 or 48 h depending on the cell tolerances to these treatments, and determined their effects on viral infectivity 48 h postinfection. We found that, indeed, ENTV entry was completely blocked by 5 or 10 nM BafA1 (p < 0.01, n = 4), and by 15 mM (p < 0.01, n = 4) NH₄Cl after the prolonged treatments (Fig. 2D and F). Similarly, a 16-h treatment with 10 or 30 μM chloroquine also significantly inhibited (p < 0.01, n = 8), but interestingly failed to completely block, the ENTV entry (Fig. 2E). Further increasing the chloroquine concentration or prolonging the treatment duration was too toxic to the cells, and the results were not informative (data not shown). It is of note that chloroquine has indeed been previously shown to have no or less inhibitory effect on entry of a number of pH-dependent viruses, including retroviruses such as equine infectious anemia virus (EIAV) [399] and foamy virus [400]. Importantly, the entry of JSRV Env and VSV-G pseudovirions was also completely blocked, or significantly inhibited, by these prolonged treatments, whereas entry of 10A1 was not affected, again demonstrating the specificities of these prolonged treatments on pH-dependent viral entry (Fig. 2D, E and F). Altogether, these results strongly suggest that viral particles accumulated in the intracellular vesicles during the 4-6 h agent treatment period remain transduction competent, and that a prolonged treatment is required for these chemicals to completely block the entry of ENTV, and perhaps JSRV as well.
**pH 4.5 or lower, but not pH 5.0, rescues a BafA1-mediated block on ENTV entry**

The requirement of an extremely low pH for fusion by ENTV Env prompted us to further examine if such a low pH is also required for overcoming a BafA1-imposed block on ENTV entry. To test this, we pre-treated HTX/LH2SN cells with 30 nM BafA1 for 1 h, and incubated cells with ENTV Env pseudovirions at 4°C for 2 h. Following extensive washes with cold PBS, the virion-cell complexes were incubated directly with a low pH solution for 5 min, or were pre-incubated at 37°C for 1 h followed by incubation with a low pH solution for 5 min. None of the low pH buffers rescued the ENTV Env pseudovirion infectivity when the cells were treated immediately after the virus binding (empty bars, Fig. 3). Notably, pre-incubation of the virion-cell complex for 1 h at 37°C followed by a treatment with pH 3.5, pH 4.0, or pH 4.5, substantially rescued the BafA1-mediated block of ENTV Env pseudovirion entry (filled bars, Fig. 3; p < 0.05 for pH 3.5, n = 3; p < 0.01 for pH 4.0, n = 5; and p = 0.054 for pH 4.5, n = 5, respectively). In contrast, a pH 5.0 treatment in parallel had no effect (p = 0.35, n = 5), similar to treatment with a pH 7.0 solution (filled bars, Fig. 3). Taken together, these results demonstrate that pH 4.5 and below, but not pH 5.0, rescues the BafA1 block of ENTV entry, further supporting the notion that the unusually low pH is necessary for ENTV Env-mediated fusion and cell entry. The greater inhibitory effect of BafA1 on ENTV entry observed here, as compared to that shown in Fig. 2A, was likely due to the removal of unbound viruses in this experiment, suggesting that constant presence of virus is another important factor that had contributed to the less inhibitory effect of BafA1 shown in Fig. 2A. Importantly, our results also indicate that a 37°C incubation period is critical for the low pH rescue of ENTV entry to
occur, presumably because that the higher temperature is required for formation of new vesicles, which, in the presence of BafA1, can only be acidified by an extracellular low pH.

**Lysosomal protease inhibitors enhance the infectivity of ENTV Env pseudovirions**

For some pH-dependent viruses, low pH-dependent lysosomal/endosomal protease activation, rather than low pH per se, is critical for membrane fusion [401-409]. Given the extremely low pH requirement for fusion of ENTV Env and the potential role of the lysosomes in this process, we next asked if lysosomal protease activities are important for ENTV Env-mediated cell entry (although the ability of ENTV Env to directly induce syncytium formation and cell-cell fusion at low pH argues against this possibility). While the infectivity of VSV-G pseudovirions was not affected by leupeptin, a broad-spectrum lysosomal protease inhibitor, the infectivity of Ebola GP pseudovirions was significantly reduced (p < 0.01, n = 3, Fig. 4A), which agreed with previous reports [401, 402]. Interestingly, ENTV Env pseudovirion infection was not inhibited, but rather, was significantly enhanced by leupeptin treatment, in a concentration-dependent manner (p < 0.05, n = 4, Fig. 4A). JSRV Env pseudovirion infection was also enhanced, but to a lesser extent (Fig. 4A). Similarly, cathepsin inhibitor III, a broad-spectrum cathepsin inhibitor, significantly inhibited Ebola GP pseudovirion infectivity (p < 0.01, n = 4), but had no significant effect on the entry of ENTV Env, JSRV Env, and VSV-G pseudovirions (Fig. 4B). Due to severe cytotoxicity of this drug, we were unable to treat cells with higher concentrations, or for prolonged periods of time, to determine if additional effects could be achieved. Nevertheless, these results show that lysosomal proteases are unlikely required for ENTV or JSRV Env-mediated entry at low pH, as opposed to other non-canonical pH-dependent viruses such as Ebola. The observed significant enhancement of ENTV
pseudovirion infectivity by leupeptin was probably due to decreased degradation of pseudoviral particles in the lysosomes where ENTV likely fuses.

**ENTV Env pseudovirions are resistant to low pH inactivation (pH 4.5 and 5.0)**

The unusually low pH-dependent fusion of ENTV Env and the possible role of lysosomes in this process also raised the question whether or not ENTV pseudovirions are stable in an acidic pH environment. To address this issue, we incubated the ENTV Env pseudovirions encoding GFP in different pH buffers at 37°C for 30 min and 5 min, and measured viral infectivity in HTX/LH2SN cells, respectively. As shown in Fig. 5, a pH 3.0 treatment for 30 min almost completely inactivated ENTV pseudovirion infectivity (p < 0.01, n = 6), similar to what was observed for JSRV [377]. Treatment at pH 3.5 or 4.0 for 30 min also substantially inactivated ENTV Env pseudovirions infectivity by ~75% and ~40% (p < 0.05, n = 6), respectively (Fig. 5). In sharp contrast, treatment at pH 4.5 or 5.0 for 30 min had no significant effect (Fig. 5) on ENTV Env pseudovirion infectivity, and this was slightly different from the situation of JSRV Env pseudovirions, whose infectivity was reduced by ~50% following treatment at pH 4.5 [377]. Noticeably, treatment of ENTV Env pseudovirions for 5 min at 37°C substantially decreased the efficiencies to low pH, in particular pH 3.0, 3.5 or 4.0, to inactivate the ENTV infectivity (Fig. 5), suggesting that longer incubation is required for these low pH to completely inactivate the ENTV infectivity. Taken together, our data show that ENTV Env pseudovirions are virtually resistant to low pH inactivation, and appear to be more stable than JSRV Env pseudovirions in acidic environments which supports its extremely low pH requirement for fusion.
However, we cannot exclude the possibility that other factors besides low pH are involved or required for the low pH inactivation to occur (see Discussion).

**The TM subunit of ENTV Env is responsible for its ability to fuse at unusually low pH, and the SU subunit contributes to its low and pH-dependent fusion activity**

We generated two chimeras between ENTV and JSRV Env in order to dissect the respective roles of the ENTV Env SU and TM in the process of low pH-dependent fusion activation. The first chimera, referred to as EJ, consisted of the ENTV SU and the JSRV TM. The second chimera, JE, consisted of the JSRV SU and the TM of ENTV. Synthesis and processing of these Env chimeras were similar to each other and to that of their parental Env molecules, according to metabolic labeling assays in 293T cells (data not shown) and immunoblotting analysis of virus-producer cell lysates (see next section, Fig. 7A).

Syncytium induction was performed to examine the fusogenicities of these chimeras relative to their parental Envs. As shown in Fig. 6A, replacement of ENTV TM with that of JSRV, as reflected in EJ, resulted in an almost 100% syncytium induction at pH 4.5, ~80% at pH 5.0, and small syncytia were still detectable at pH 6.0, a pattern similar to that of JSRV Env. Of note, the percentages and the sizes of syncytia induced by EJ generally were less than those induced by JSRV Env, particularly at pH 4.5 and above (Fig. 6A), suggesting that the SU subunit of ENTV Env influences its fusogenicity. Conversely, replacement of JSRV TM with that of ENTV, as reflected in JE, essentially blocked the fusion activity of JSRV Env at pH 5.0, but converted fusion at pH 4.0 or 4.5, a pattern which was similar to that of ENTV Env (Fig. 6A). Again, the fusion activity of JE was higher than of that of ENTV Env under these low pH conditions (pH 4.0 and 4.5) (Fig. 6A).
To confirm the above results and better quantify the fusion activities of these chimeric Envs relative to their parental Env, cell-cell fusion assays were carried out in the presence of pH 4.0, 5.0, or 7.0. No cell-cell fusion was detected for ENTV Env at pH 5.0 following a 1 min treatment (Table 1 and Fig. 6B), consistent with the results of syncytium induction assay shown in Fig. 1 and Fig. 6A. Interestingly, we only detected a very low level of cell-cell fusion activity at pH 4.0 (Table 1 and Fig. 6B), compared to the substantial syncytium formation at pH 4.0 shown in Fig. 1A and Fig. 6A. In contrast, chimeric EJ, containing the JSRV TM, showed significantly higher fusogenicity than did the parental ENTV Env at both pH 5.0 (p = 0.003, n = 4) and pH 4.0 (p = 0.028, n = 4) (Fig. 6B). Yet, the fusion activity of EJ was still lower than that of JSRV Env (p = 0.01 and 0.04 for pH 4.0 and pH 5.0, respectively, n = 4; Fig. 6B). In agreement with the syncytium induction assay (Fig. 6A), chimeric JE, containing the ENTV TM, failed to induce cell-cell fusion at pH 5.0, and also exhibited a much less fusion activity at pH 4.0 than did the parental JSRV Env (p = 0.01, n = 4; Fig. 6B). We noticed that the fusion activity of JE was still higher than that of ENTV Env at pH 4.0 (p = 0.02, n = 4; Fig. 6B). Altogether, our data suggest that the TM subunit of ENTV Env is responsible for its unusually low pH-dependent fusion activity.

Flow cytometric analysis of a portion of 293T/GFP effector cells revealed that surface expression of the SU of EJ was in fact higher than that of JSRV Env, but was comparable to that of ENTV (Fig. 6C). By contrast, surface expression of the SU of JE was less than that of ENTV Env, yet was similar to that of JSRV Env (Fig. 6C). These results indicated that the lower fusogenicity of EJ than JSRV Env, and of ENTV Env than JE, were not due to Env expression on the cell surface. Collectively, these results revealed that the TM subunit of ENTV Env is responsible for its unusually low pH-dependent fusion activity.
activation, and that the SU subunit of ENTV Env also critically influences its low and pH-dependent fusogenicity.

**Incorporation of ENTV Env and its chimeras into a MoMLV retroviral vector and transduction efficiency of cells expressing different levels of Hyal2**

Previous studies showed that ENTV has limited host range compared with JSRV, based on the finding that a MoMLV vector bearing ENTV Env did not effectively transduce cell lines derived from most species, including humans and sheep [369]. To determine if this is due to a defect in the incorporation of ENTV Env into the MoMLV vector, or to other factors, such as receptor binding or possibly fusion, here we generated alkaline phosphatase (AP)-encoding MoMLV pseudotypes bearing ENTV Env, JSRV Env, or their chimeras, and examined Env expression, incorporation, and vector transduction efficiencies in cells expressing different levels of Hyal2.

Env expression in virus-producer cells and in viral particles was examined by immunoblotting using an anti-FLAG antibody. As shown in Fig. 7A, ENTV Env and its two chimeras were expressed efficiently and processed properly into SU and TM in the virus-producer cells, with efficiencies similar to that of JSRV Env. Of note, ENTV Env, in particular the SU subunit, migrated slightly slower than JSRV Env, likely due to their differences in molecular weight and post-translational modification (e.g., there is one extra potential N-linked glycosylation site in the ENTV Env SU than in the JSRV Env SU) (Fig. 7A). Interestingly, the signal for the SU subunit of JSRV Env derived from cell lysates was consistently weaker than that of ENTV Env (Fig. 7A), which was in parallel with its relatively low surface expression 293/LH2SN and 293T/GFP cells (Fig. 1C, and 6C). The reason behind the low JSRV SU relative to that of ENTV were not investigated further, but
might be associated with the different trafficking and endocytosis signals present in their respective Env CTs [381].

We found that ENTV Env was incorporated into the MoMLV vector as efficiently as was JSRV Env (Fig. 7B), although this was not counting the relatively low levels of JSRV Env surface expression (~50%) compared to ENTV Env (Fig. 1C and 6C). Indeed, ~3-fold more EJ was incorporated than ENTV Env, and ~2-fold less JE was incorporated than JSRV Env, in the pseudovirion particles, based on quantitative analyses of Env and MoMLV Gag protein signals detected by immunoblotting (Fig. 7B). These data suggest that the TM subunit of ENTV Env is not as compatible as that of JSRV Env for its incorporation into the MoMLV vector, as has been previously suspected (14).

The transduction efficiency of MoMLV vectors bearing individual Envs was determined in several cell lines. In HTX cells expressing endogenous Hyal2, transduction by ENTV Env pseudovirions was extremely poor, with ~26 AP+ foci per ml, which was over 1000-fold lower than that of JSRV Env (Table 2). Replacement of ENTV Env TM with that of JSRV Env (as reflected in EJ) increased the titer by ~6-fold (Table 2), similar to a previous report [369, 383]. Impressively, substitution of ENTV SU with that of JSRV (as reflected in JE) significantly increased the ENTV titer to ~10^4 AP+ foci per ml, a level nearly comparable to that of JSRV Env (Table 2). In HTX or mouse NIH 3T3 cells that over-express human Hyal2, the titers of all Env pseudovirions were significantly increased, particularly those bearing ENTV Env and EJ (Table 2). Collectively, these results reveal that ENTV Env SU-Hyal2 interaction is likely the key determinant for the low infectivity of ENTV Env pseudovirions in HTX and most other cells expressing endogenous Hyal2.

Noticeably, EJ exhibited a titer that was approximately 10-20-fold higher than that of ENTV, and JE exhibited a titer that was ~5-10-fold lower than that of JSRV Env in all
three cell lines tested (Table 2). These titer differences cannot be simply explained by a 2-3-fold decrease in Env incorporation shown in Fig. 7B. One possible explanation is that Env pseudovirions containing the ENTV TM (i.e., JE and ENTV) might be more prone to lysosomal degradation due to their extremely low pH requirement for fusion, compared to those of Env pseudovirions containing the JSRV TM (i.e., JSRV and EJ). Indeed, treatment of HTX/LH2SN cells with 50 μM leupeptin substantially increased the titer of JE pseudovirions by ~33%, a degree that was similar to that of ENTV Env pseudovirions (Fig. 4A), while only increasing the titer of EJ pseudovirions by ~10%, which was comparable to that of JSRV Env pseudovirions (Fig. 4A). These data therefore support the notion that the TM subunit of ENTV Env can modulate the vector transduction efficiency not only by slightly reducing the Env incorporation into the MoMLV vector but also by affecting the pH-dependent fusion process.

3.6 Discussion

We show in this study that the fusogenicity of ENTV Env protein is much lower than that of JSRV Env and it requires a more acidic pH for fusion activation and cell entry (Fig. 1, Fig. 6, and Table 1). The threshold pH value that activates ENTV Env-mediated fusion was estimated to be ~4.5, which is much lower than that of JSRV Env (~pH 6.0) (Fig. 1 and Fig. 6), and other typical pH-dependent viral glycoproteins (pH 4.8-6.5) [410], such as influenza virus haemagglutinin (HA) (pH 5.7) [411, 412], VSV-G (pH 6.3) [413-415] and Semliki Forest virus (SFV) E1 (pH 6.2) [415, 416]. While the cell-based fusion assays do not always mimic productive viral infection [417] and it is possible that ENTV may fuse with cell membrane at pH 5.0 during infection in vivo, the differences between
ENTV and JSRV Env proteins in fusogenicity as well as in low pH requirements for fusion activation in vitro are intriguing. It would be ideal to confirm the results shown in this study with an infectious ENTV clone, however, a tissue culture system that can produce high titers of ENTV is not currently available which would allow us to perform such related experiments. The ENTV Env used in this study was derived from the first full length ENTV strain (also called ENT1V1, GenBank Y16627 and AF401741), which is considered to represent all other ENTV strains isolated thus far [369, 379, 383]; however, we cannot rule out the possibility that other ENTV Env isolates may have somewhat different fusion activities and low pH requirements.

The low fusogenicity of ENTV Env and its particular requirement for a very acidic pH for fusion are not due to abnormal Env trafficking, processing or expression. In fact, the ENTV Env protein was synthesized and processed very efficiently (Fig. 7A), with consistently higher SU surface expression (Fig. 1C and Fig. 6C) and basal levels of SU secretion into culture medium than that of JSRV Env (data not shown). Using chimeras between JSRV and ENTV Env, we demonstrate that the TM subunit of ENTV Env is likely responsible for the extremely low pH requirement and largely accounts for its low fusion activity (Fig. 6A and 6B). This finding is consistent with the general notion that low pH acts directly on the TM subunits of pH-dependent retroviral Env, triggering a series of conformational rearrangements that are required for membrane fusion [417, 418]. It is also of note that the major difference between ENTV and JSRV Env is located in the TM subunit, with ~82% amino acid identity in this region compared to an overall ~89% amino acid identity in the full length Env [367, 369, 379]. Future efforts will be aimed at determining what regions or specific domains of the ENTV Env TM subunit are
responsible for the extremely low pH requirement for fusion and investigating the underlying mechanisms.

We demonstrated in this study that cell entry mediated by ENTV Env is also pH-dependent, but found, unexpectedly, that the patterns of ENTV entry are quite different from that of JSRV. First, pH 4.0 or 4.5, but not pH 5.0, substantially overcame a BafA1-mediated block of ENTV entry (Fig. 3), while the BafA1-mediated block of JSRV entry was readily rescued by treatment at pH 5.0 [377]. Second, although the infectivity of ENTV Env pseudovirions was inhibited by BafA1, the extent of this inhibition was consistently lower than seen with JSRV during the 6 h incubation period (Fig. 2A). Moreover, the inhibition was not concentration-dependent, which was also different from that of JSRV and VSV (Fig. 2A). Third, NH₄Cl, and to a lesser extent, chloroquine, were not able to inhibit, but rather substantially enhanced, the entry of ENTV Env pseudovirions following a 4 h treatment period (Fig. 2B and C). Again, this was in sharp contrast to JSRV, whose infection was substantially inhibited by similar treatments (Fig. 2B and C). Lastly, treatment of cells with leupeptin, a broad-spectrum lysosomal protease inhibitor, appeared to enhance ENTV entry more significantly than JSRV entry (Fig. 4A). Taken together, our data strongly suggest that the endocytic compartments that mediate the fusion of ENTV Env are different from those required by JSRV Env. For example, ENTV Env-mediated fusion and cell entry may predominantly occur in more downstream endocytic compartments, such as lysosomes (~pH 4.0-5.0), while JSRV Env fusion may predominantly occur in late endosomes (~pH 5.0-6.0). As such, ENTV Env pseudovirions may be more prone to lysosomal degradation, compared to those of JSRV Env, and this would explain the significant enhancement of ENTV infectivity by leupeptin, relative to that of JSRV (Fig. 4A), and also explain, at least partially, the differential effects of BafA1
and lysosomotropic agents on ENTV and JSRV infection during the 4-6 h treatment period (Fig. 2A-C). However, it should be emphasized that the less inhibitory effect of BafA1 and apparent enhancement of lysosomotropic agents on ENTV infection following the shorter (4-6 h) incubation were also attributable to, and in conjunct with, other factors, such as the constant presence of viruses with the agent (Fig. 2A-C), as well as the ability of ENTV Env pseudovirions to resume transduction upon withdrawal of these chemicals (Fig. 2D-F).

While virus fusion in cellular lysosomes is conceivably not advantageous for viral fitness, it is noticeable that a number of classical pH-dependent enveloped viruses, including influenza [419, 420], and some non-canonical pH-dependent viruses, such as Ebola, severe acute respiratory syndrome coronavirus (SARS-CoV), and MoMLV which require lysosomal/endosomal protease activity for fusion [401-405, 409], have been shown, or implicated, to use lysosomes and/or late endosomes to enter host cells. In this regard, it will be interesting to determine if ENTV Env-mediated fusion truly occurs in lysosomes, or if other endocytic vesicles, such as late endosomes, are also involved in this process. Ultimately, the exact entry pathways of ENTV and JSRV will need to be elucidated.

For a typical pH-dependent virus, viral infectivity should be inactivated by low pH because of premature conformational changes that can block further infection [417]. However, we found in this study that ENTV Env pseudovirions are quite resistant to low pH inactivation. For example, the infectivity of ENTV Env pseudovirions only decreased slightly after 30 min in a pH 4.5 or 5.0 buffer at 37°C (Fig. 5). This was in contrast to that of JSRV whose infectivity was reduced by almost 50% at pH 4.5 [377]. While these results may be used to argue against the pH-dependent fusion of ENTV Env, we cannot rule out the possibility that there is still a pre-triggering at pH 4.0 and below (Fig. 5) and that the conformational changes of ENTV Env induced by low pH is reversible, as has been shown.
for VSV-G [421]. Alternatively, a receptor binding step may be required for the low pH inactivation to occur; and if this is true, ENTV would resemble avian sarcoma and leukosis virus (ASLV), which uses a two-step fusion process [422]. Although we favor the latter explanation, given some additional lines of evidence discussed below, we cannot rule out the first possibility.

The two-step fusion model described for ASLV states that a specific interaction between ASLV Env and its corresponding receptor induces the initial conformational changes of Env that are required to prime the subsequent low pH trigger [422]. We show here that, while the TM subunit of ENTV Env plays a major role in dictating the extremely low pH requirement for fusion and therefore largely accounts for its low fusion activity, the SU subunit of ENTV Env also critically modulates its low and pH-dependent fusion activities (Fig. 6A and B; Table 1). This raises the possibility that ENTV Env SU-Hyal2 interaction may regulate the pH-dependent fusion process. Indeed, the binding affinity of ENTV SU for human Hyal2 has been shown to be much lower than that of JSRV SU for human Hyal2 (~175 nM for ENTV versus ~10 nM for JSRV) [352, 383], which may explain the relatively low fusogenicities of Env constructs harboring the ENTV Env SU (Fig. 6). However, we cannot rule out the possibility that other regions of ENTV SU not directly involved in receptor binding, such as the proline-rich motifs, may also regulate fusion, as has been shown for MLV [423]. In addition, we have attempted to detect oligomerization of ENTV Env in the viral particles at low pH, but repeatedly failed to observe formation of high-molecular weight species by SDS-PAGE (data not shown), again suggesting that other factors besides low pH might be involved in the low inactivation process. Taken together, we propose that Hyal2 may play a very important role in the pH-dependent fusion of ENTV Env (and JSRV Env as well); however, additional experiments
are required to critically test this hypothesis. Whether or not ENTV utilizes a two-step process for fusion and cell entry as ASLV will be explored in future studies.

3.7 Acknowledgement

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Table 3.1. Fusion activity of ENTV Env, JSRV Env or their chimeras

<table>
<thead>
<tr>
<th>Env</th>
<th>Fusion (%)</th>
<th>pH 4.0</th>
<th>pH 5.0</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENTVC</td>
<td>2.22</td>
<td>1.78</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>JE</td>
<td>11.12</td>
<td>7.25</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>JSRV</td>
<td>15.07</td>
<td>10.32</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>JE</td>
<td>5.73</td>
<td>1.96</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.42</td>
<td>0.42</td>
<td>0.61</td>
<td></td>
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</tbody>
</table>

Effector 293T/GFP cells were transfected with plasmids encoding individual Env proteins; 24 h post-transfection, cells were split and co-cultured for 1 h with HTX/LH2SN target cells that were pre-labelled with CMTMR. Co-cultured cells were treated with different pH buffers for 1 minute, and the percentages of fused cells (%) were determined by flow cytometry after a 1-h recovery. Experiments were performed in duplicate, and one representative experiment is shown. Values are averages of two duplicate samples. See Fig. 6 legend for nomenclatures and Fig. 6B for a comprehensive summary of results from multiple experiments.
Table 3.2. Titers of MoMLV pseudovirions bearing ENTV Env, JSRV Env, or their chimeras in cells expressing different levels of Hyal2 (AP+ foci per ml)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pseudotyping Env</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ENTV</td>
</tr>
<tr>
<td>HTX</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>HTX/LH2SN</td>
<td>(1.8 ± 0.7) × 10⁵</td>
</tr>
<tr>
<td>NIH 3T3/LH2SN</td>
<td>1.5 × 10⁴</td>
</tr>
</tbody>
</table>

293/GP-LAPSN cells expressing the MoMLV Gag-Pol proteins and alkaline phosphatase (AP) were transfected with a plasmid encoding each Env, and viral supernatants were harvested 48-72 h post-transfection. Cells were infected with appropriate amounts of viral stocks in the presence of polybrene, and AP+ foci were determined ~72 h post infection. Results are averages of 2-3 independent experiments ± standard deviations.
Fig. 3.1. **ENTV Env requires a very low pH to induce cell fusion.** (A) Syncytium induction. 293/LH2SN cells co-transfected with plasmids encoding ENTV Env and GFP were treated with a pH solution ranging from 4.0 to 7.0 for 1 min (upper panel) or 5 min (lower panel), and monitored for syncytium formation over time. Pictures were typically taken 1 h after the pH treatment using a fluorescence microscope. Representative pictures from at least 4 independent experiments are shown. (B) Cell-cell fusion. 293T/GFP cells transfected with a plasmid DNA encoding ENTV Env, JSRV Env, or VSV-G were co-cultured with HTX/LH2SN that were pre-labelled with CMTMR. Cells were treated with a pH solution ranging from 3.0 to 7.5 for 1 min, and analyzed for fusion by flow cytometry following a 1 h recovery period. For each fusion protein, the highest fusion activity at an optimal pH was set to 100%, and the fusion activities at other pH conditions were calculated relative to the highest values, and were plotted against the pH values used. Results are averages of at least three independent experiments ± standard deviations. (C) Env surface expression. Cells were stained with an anti-FLAG mouse antibody, followed by incubation with a FITC-(293/LH2SN) or PE (293T/GFP)-labelled anti-mouse antibody, and analyzed by flow cytometry. Solid lines represent the fluorescence shifts of ENTV Env, broken lines represent the fluorescence shifts of JSRV Env, and filled areas represent cells incubated with secondary antibody alone. A representative of three independent experiments is shown.
Fig. 3.2. Effects of bafilomycin A1 (BafA1), chloroquine, and ammonium chloride (NH₄Cl) on ENTV entry. (A-C) HTX/LH2SN cells were pre-treated with BafA1 (A) for 2 h, chloroquine (B) or NH₄Cl (C) for 1 h at indicated concentrations, and were infected with GFP-encoding pseudovirions bearing ENTV Env, JSRV Env, VSV-G, or amphotropic 10A1 MLV Env in the presence of respective agents for 6 h (A) or 4 h (B and C). (D-F) HTX/LH2SN cells were pre-treated with BafA1, chloroquine, or NH₄Cl at indicated concentrations for 1 h, and infected with each pseudotype in the presence of agents for 16 h (D and E) or 48 h (F). In all cases, viral infectivity was determined 48 h postinfection by flow cytometry. Percent infections were calculated relative to untreated cells, and values are averages of 2-8 independent experiments ± standard deviations. Paired two-tail student’s t tests were used for statistical analysis; * indicates p < 0.05, and ** indicates p < 0.01.
Fig. 3.3. pH 4.5 or below rescues a BafA1-imposed block on ENTV entry. HTX/LH2SN cells pre-treated with BafA1 were incubated with GFP-encoding pseudovirions bearing ENTV Env for 2 h at 4°C. Unbound pseudovirions were removed by washing cells with cold PBS; and virus-bound cells were either directly treated with different pH buffers for 5 min (empty columns, “no incubation”), or alternatively, were pre-incubated at 37°C for 1 h in the presence of BafA1, followed by treatment with different pH buffers for 5 min (filled columns, “1 h incubation”). Note that in both cases, the total infection period at 37°C was 4 h in the presence of BafA1. The percent infections were calculated relative to that of the untreated/pH 7.0-treated cells, and values are averages of 3-6 independent experiments ± standard deviations. Note that the experimental conditions used here (unbound virus was removed after the 2 h binding step) was different from that used in Fig. 2A (pseudovirions were constantly present throughout the 6 h infection/treatment period), which likely accounted for the more inhibitory effect of BafA1 shown in this figure. * indicates p < 0.05, and ** indicates p < 0.01.
Fig. 3.4. Effects of lysosomal protease inhibitors on ENTV entry. HTX/LH2SN cells were pre-treated with leupeptin (A) or cathepsin inhibitor III (B) at indicated concentrations for 1 h, and infected with GFP-encoding pseudovirions bearing ENTV Env, JSRV Env, VSV-G, or Ebola GP in the presence of each drug for 3 h (A) or for 4 h (B). The percent infections were calculated relative to untreated cells, and values are averages of 2-4 independent experiments ± standard deviations; * indicates p < 0.05, and ** indicates p < 0.01.
Fig. 3.5. ENTV Env pseudovirions are resistant to low pH inactivation. GFP-encoding MoMLV pseudovirions bearing ENTV Env were treated with different pH solutions ranging from 3.0 to 7.0 at 37°C for 30 or 5 min. Following neutralization with culture media, virions were used to infect HTX/LH2SN, and percentages of infected cells were measured 48 h post-infection by flow cytometry. Viral infectivity (%) at each pH was calculated relative to the pH 7.0-treated viral stocks (set to 100%); the results are averages of 4 or 6 independent experiments ± standard deviations. * indicates p < 0.05, and ** indicates p < 0.01.
Fig. 3.6. Fusogenicities of ENTV Env chimeras in comparison with those of parental ENTV and JSRV Env. “EJ” consists of the ENTV SU and the JSRV TM, while “JE” consists of the JSRV SU and the ENTV TM. (A) Syncytium induction assay was performed as described in Fig. 1A, except that different Env-encoding plasmids were used to transfect 293/LH2SN cells, and that cells were treated with indicated pH for 1 min. Results are representatives of 3-5 independent experiments. (B) Cell-cell fusion was performed as described in Fig. 1B, except that different Env-coding plasmids were tested under three indicated pH conditions. The relative fusion activities of each Env at different pH were calculated relative to that of JSRV Env at pH 5.0 (set to 100%, the dashed line). Due to complex comparisons, the $p$ values were not labeled in this graph, but were instead provided in the relevant text. Also note that the $p$ values were not corrected by Bonferroni adjustment because in this particular case only two Env proteins of interest were compared to each other. No-Env: parental 293T/GFP cells with no plasmid being transfected. (C) Env surface expression in 293T/GFP cells was determined by flow cytometry as described in Fig. 1C, with geometric fluorescence means being acquired for each Env. All values were normalized to that of the parental ENTV Env, whose geometric mean was set to 100%. Averages of 4-6 independent experiments ± standard deviations are shown for both (B) and (C).
Fig. 3.7. Env expression of ENTV, JSRV, and their chimeras in virus-producer cells. (A) and in pseudovirion particles (B). 293/GP-LAPSN cells were transfected with a plasmid encoding each Env, and supernatants containing pseudovirions were harvested 48-72 h post-transfection. (A) Cell lysates were analyzed by immunoblotting using anti-FLAG antibody (upper panel); the same membrane was then stripped, and re-blotted using anti-β-actin (lower panel). (B) Pseudovirions were purified on sucrose cushion, and Env incorporation was analyzed by immunoblotting using an anti-FLAG antibody (upper panel). Membrane was stripped, and re-blotted with anti-MLV gag (lower panel). Densitometric quantification of TM (upper) or gag (lower) bands was performed using the Quantity One software (Bio-Rad), and the relative intensities were obtained by setting the signals of JSRV Env to 1.00. No-Env: parental 293/GP-LAPSN cells with no plasmid being transfected. Representative results of 3 to 4 independent experiments are shown.
CHAPTER 4

RECEPTOR BINDING AND LOW pH COACTIVATE ONCOGENIC RETROVIRUS

ENVELOPE-MEDIATED FUSION

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4.1 Preface to Chapter 4

We have shown in Chapter 2 that Hyal2 overexpression is required for JSRV Env-mediated cell-cell fusion. Additionally, we clearly showed in Chapter 3 that SU is able to modulate Env fusion activity. These two findings suggest that receptor binding plays an active role in fusion activation. This is in contrast to the common belief that receptor binding only serves as a mean to enter the cell by endocytosis and mainly plays a passive role in the fusion mechanism of pH-dependent viruses. In order to further investigate the role of the receptor during fusion, we generated a soluble form of the receptor that we used in our fusion assays, which enabled us to pinpoint the step(s) of the JSRV Env fusion process that require the presence of the receptor. To further investigate the conformational rearrangement of the TM subunit during fusion activation, we developed a new assay in which the different oligomeric states of TM could be visualised. Interestingly, such an assay exists only for ASLV Env and is based on the intrinsic high stability of the post fusion conformation, resisting mild SDS treatment. However, we discovered that the activated JSRV Env was extremely sensitive to SDS treatment, preventing us from using an assay similar to that of ASLV Env. Therefore, based on a recent study on MLV Env activation, we used a cross-linker to fix the oligomeric activated Env. This technique enabled us to investigate the role of Hyal2 in the fusion mechanism of JSRV Env presented in this chapter.
4.2 Abstract

Fusion of enveloped viruses with host cells is triggered by either receptor binding or low pH, but rarely requires both except for avian sarcoma leukosis virus (ASLV). We previously reported that membrane fusion mediated by an oncogenic jaagsiekte sheep retrovirus (JSRV) envelope (Env) requires an acidic pH, yet observed that receptor overexpression is required for this process to occur. Here we show that a soluble form of the JSRV receptor, sHyal2, promoted JSRV Env-mediated fusion at low pH in normally fusion-negative cells, and this effect was blocked by a synthetic peptide analogous to the C-terminal heptad repeat of JSRV Env. Remarkably, severe truncation of JSRV Env cytoplasmic tail resulted in membrane fusion at neutral pH, for which receptor overexpression was no longer required. Distinct from that of ASLV, sHyal2 induced pronounced shedding of surface (SU) subunit, as well as unstable conformational rearrangement of the transmembrane (TM) subunit, yet full activation of JSRV Env fusogenicity, associated with strong TM oligomerization, required both sHyal2 and low pH. Consistently, sHyal2 enabled transduction of nonpermissive cells by JSRV Env pseudovirions, with low efficiency, but substantially blocked viral entry into permissive cells at both binding and post-binding steps, indicating that sHyal2 prematurely activates JSRV Env-mediated fusion. Altogether, our study supports for a model that receptor priming promotes fusion activation of JSRV Env at low pH, yet the underlying mechanism appears to be distinct from that of ASLV. Hence, JSRV may provide an alternate useful model for better understanding of virus fusion and cell entry.
4.3 Introduction

Fusion is a fundamental event in the life cycle of enveloped viruses, and is essential for viral replication. While highly divergent in primary sequence, the structures of viral fusion proteins and their modes of activation share striking similarities, permitting classification of viral fusion proteins into two major groups [424]. Class I fusion proteins, as exemplified by the retrovirus envelope (Env) and influenza virus hemagglutinin (HA), are composed mainly of alpha helices, and are present as metastable trimers on the viral surface [418]. Class II fusion proteins, represented by alphavirus E1 and flavivirus E, contain predominantly beta-sheets and exist as heterodimers in the pre-fusion state [116]. Of note, the vesicular stomatitis virus G (VSV-G) and herpesvirus gB proteins were recently assigned to a newly established class III, for fusion proteins combining properties of both class I and class II [111, 425]. Despite these differences, one common and intriguing characteristic of all viral fusion proteins is their ability to undergo conformational rearrangements upon activation, i.e., the formation of trimers of hairpins, which drive membrane fusion between viral and cellular membranes [37, 418].

Retrovirus Env is a typical type I transmembrane protein composed of surface (SU) and transmembrane (TM) subunits. SU is responsible for binding to cognate cellular receptors or cofactors, while TM directly mediates membrane fusion [426]. Most retroviruses use a pH-independent pathway for entry, during which receptor binding relieves the ability of SU to restrain TM, resulting in conformational changes in TM and subsequent fusion with the cell membrane [418]. Interestingly, increasing numbers of retroviruses have recently been shown to require a low pH or pH-dependent protease activities to trigger fusion [399, 400, 405, 422, 427, 428]. Among these, avian sarcoma
leukosis virus (ASLV) represents a unique example, as it uses a two-step mechanism for fusion, in which receptor binding primes the second trigger of low pH [422].

Jaagsiekte sheep retrovirus (JSRV) is a simple betaretrovirus etiologically responsible for a contagious lung tumor in sheep [366]. The native Env protein of JSRV functions as a potent oncogene that induces cell transformation in vitro and in animals [370, 371, 374, 375, 429]. The cell entry receptor for JSRV has been identified as hyaluronidase 2 (Hyal2), a glycosylphosphatidylinositol (GPI)-anchored protein belonging to the hyaluronidase family [371], although Hyal2 itself has low hyaluronidase activity, and this activity is not directly associated with JSRV entry and infection [430]. Intrigued by the oncogenic nature of JSRV Env, we recently examined the mechanism of JSRV entry, and found that JSRV Env-mediated fusion and cell entry requires a low pH [431, 432]. These observations led us to hypothesize that the pH-dependent fusion activation of JSRV Env may be advantageous for its oncogenesis, given that extreme cell-cell fusion of the plasma membrane at neutral pH would result in syncytia formation and often cell death. Curiously, we noticed that overexpression of Hyal2 is necessary for JSRV Env to induce membrane fusion in vitro at low pH, suggesting that Hyal2 may play an active role in the pH-dependent fusion process. Here we provide direct evidence that Hyal2 functions in cooperation with low pH to trigger JSRV Env-mediated fusion activation, yet exhibits some striking differences from that of ASLV. The multistep pathway for JSRV Env-mediated fusion activation might be important for its replication fitness and oncogenesis.
4.4 Materials and Methods

Cell lines, viruses and reagents. All mammalian cells used this study were maintained in DMEM supplemented with 10% FBS using standard procedures. Drosophila Schneider 2 (S2) cells were grown at 27°C in Express Five SFM media (Invitrogen, Carlsbad, CA) supplemented with 10% L-Glutamine (Invitrogen). MoMLV pseudotypes bearing JSRV Env (with a FLAG tag at both N- and C-termini) or VSV-G were produced as previously described [431]. All primary and secondary antibodies were purchased from Sigma (St. Louis, MO). The peptides corresponding to the JSRV N-terminal heptad repeat (N-HR1) (DKKIEDRLSALYDVVRVLGE) and C-terminal heptad repeat (C-HR2) (FNTNLSLDLQLHNEILDIENS) were synthesized by Alpha Diagnostic International (San Antonio, TX), and were solubilized in water and reconstituted in 14% dimethyl sulfoxide (C-HR2 is insoluble in water), respectively.

Soluble Hyal2 production and purification. Drosophila S2 cells stably expressing sHyal2 (kind gift of Vlad Vigdorovich and Dusty Miller) were induced by 1 mM CuSO4 for 5-7 days at 27°C, culture media were harvested, and sHyal2 protein was purified using Ni-NTA columns (Qiagen, Valencia, CA). The purity of sHyal2 was determined by SDS-PAGE, and quantified by Bradford assays.

Cell surface staining. Cell surface staining was performed as previously described [352], except that an anti-FLAG antibody, or 0.5 µg of sHyal2 followed by an anti-His antibody, was incubated with 293 cells expressing FLAG-tagged JSRV Env, followed by flow cytometry analysis.
Syncytia induction assay. Syncytia induction assays were performed as described previously [431], with minor modifications. Briefly, 293 cells were co-transfected with plasmids encoding JSRV Env and pCMV-GFP; 24 h post-transfection, cells were incubated with different amounts of sHyal2 for 1 h at 4°C, followed by incubation at 37°C for 30 min. Cells were then treated with pre-warmed pH 7.4 or pH 5.0 buffer for 5 min, incubated for 1 h at 37°C before being examined for fusion, and photographed. For syncytia induction involving heptad repeat peptides, transfected cells were incubated with a peptide or sHyal2 for 1 h at 4°C, followed by incubation at 37°C for 30 min; cells were then incubated with fresh media containing either sHyal2 or the peptide at 4°C for 1 h, followed by recovery at 37°C for 30 min. The sHyal2-peptide treated cells were incubated with a pH 5.0 buffer for 5 minutes, and analyzed for fusion using fluorescence microscopy.

Metabolic labelling. Metabolic labeling was performed as described previously [431], except that different amounts of sHyal2 were incubated with 293 or 293/LH2SN cells during the last 3 h of a 6-h chase period. Cell lysates and culture media containing the $^{35}$S-Met/Cys-labelled JSRV Env were harvested, and immunoprecipitated using anti-FLAG beads, respectively, and samples were boiled in buffer containing 1% SDS and 1% beta-mercaptoethanol for 10 min, and resolved by SDS-PAGE.

Oligomerization assay. JSRV Env pseudovirions were pelleted by centrifugation for 3 h at 185,000 × g on a 1.5-ml 20% sucrose cushion and resuspended in PBS. Purified pseudovirions were incubated with appropriate concentrations of sHyal2 or equal volumes of PBS on ice for 30 min, followed by incubation at 37°C for 30 min. The complex was then treated with a pH 7.4 or 5.0 buffer (acidified with a pre-determined volume of 0.1N
HCl) for 5 min at 37°C, and neutralized with 0.1 N NaOH if necessary. Unless otherwise stated, the complex was crosslinked by 25µM Dithiobis(succinimidylpropionate) (DSP) (Pierce, Rockford, IL) for 30 min at room temperature, with remaining reactive DSP quenched by ~0.2 M Tris (pH 8.0). For oligomerization assays involving different temperatures, the virion-sHyal2 complex was incubated 5 min at the indicated temperatures before being crosslinked with 25 µM DSP. To assess the stability of the oligomers, the virion-sHyal2 complex, treated with either a pH 7.4 or pH 5.0 buffer, was incubated with indicated concentrations of SDS or urea for 5 min at 37°C. All samples were incubated in a non-reducing sample buffer containing 0.1% SDS for 5 min at 37°C before being resolved by 7.5% SDS-PAGE, followed by western blot as previously described [386].

**Infection.** For infection of non-permissive cells, GFP-encoding MoMLV pseudoparticles bearing JSRV Env or VSV-G were bound to HeLa cells in DMEM containing 5 µg/ml polybrene by spinoculation at 1,680 × g for 2 h at 4°C. Appropriate amounts of sHyal2 were incubated with the cells for 48 h before the cells were analyzed by flow cytometry. To assess the effect of sHyal2 on permissive cells, HTX cells were pre-bound by virions in media containing polybrene for 1 h at 4°C; cells were washed to remove unbound virions, and incubated with sHyal2 for 1 h at 4°C. Pseudovirions were incubated with appropriate amounts of sHyal2 on ice for 30 min, and the virus-sHyal2 complex was added to HTX for binding at 4°C for 2 h; unbound viruses were removed, and cells were incubated for 48 h before analysis using flow cytometry to measure the percentages of GFP positive cells. The relative rate of infection was calculated against cells that had not been treated with sHyal2.
4.5 Results

Soluble Hyal2 (sHyal2) mediates syncytia induction by JSRV Env at low pH

To address the possible role of Hyal2 in JSRV Env-mediated fusion, we produced a soluble form of Hyal2 (sHyal2) using a Drosophila S2 cell line that stably expresses Hyal2 (kindly provided by Drs. Vlad Vigdorovich and Dusty Miller). In this system, the GPI anchor of Hyal2 was replaced by a histidine tag, and the expressed proteins were secreted into cell culture medium. We used Ni-NTA columns to isolate sHyal2 to high purity (>95%) (Fig. 1A), and its ability to interact with JSRV Env was determined by flow cytometry, which demonstrated specific binding of sHyal2 to 293 cells expressing a FLAG-tagged JSRV Env (Fig. 1B).

We first examined whether sHyal2 could induce membrane fusion of JSRV Env in 293 cells at low pH. In the absence of sHyal2, no syncytia were observed (Fig. 1C), as we previously reported [431]. Remarkably, preincubation of the Env-expressing 293 cells with 1.5 or 15 µg/ml of sHyal2 for 1 h, followed by a pH 5.0 pulse for 5 min, led to pronounced syncytia formation, though syncytia formation did not occur in a strictly dose-dependent manner (Fig. 1C, and data not shown). The concentration of sHyal2 required to induce syncytia under these conditions (pH 5.0 for 5 min) was ~1 µg/ml. In contrast, cells preincubated with 15 µg/ml of sHyal2, followed by a neutral-pH pulse, showed no apparent fusion (Fig. 1C). A JSRV Env chimera that contains the SU from a closely related enzootic nasal tumor virus (ENTV), resulting in reduced binding to Hyal2 [433], required increased concentrations of sHyal2 (>10 µg/ml) to induce fusion (Fig. 1D). The reduced fusogenicity of this chimera (referred to as EJ in ref. 20) was not a result of lower Env expression, as the chimera exhibited slightly greater Env expression than did wildtype Env [433]. Together,
these results demonstrate that Hyal2 is required to induce fusion of JSRV Env at low pH, most likely by acting through its interaction with the JSRV SU.

The ability of sHyal2 to induce membrane fusion at low pH is blocked by a C-heptad repeat peptide of JSRV Env

We next sought to determine the step(s) of the fusion process at which Hyal2 is required for syncytia formation by JSRV Env in 293 cells. To this end, we incubated Env-expressing 293 cells with sHyal2 before, during, or after pH 5.0 treatment, and examined the effects of sHyal2 and low pH on Env-mediated fusion induction. Incubation of sHyal2 after or during low pH treatment induced no or mild fusion, whereas incubation of sHyal2 prior to low pH treatment, or inclusion of sHyal2 in the entire fusion assay, led to robust syncytia formation with an increased number and size of syncytia (Fig. 1E). Remarkably, a synthetic peptide analogous to the C-terminal heptad repeat (C-HR2) of JSRV Env, but not to that of N-terminal HR (N-HR1) (data not shown), substantially blocked syncytia formation (Fig. 1F). The effect of JSRV C-HR2 on the Env-mediated fusion was most pronounced when the peptide was added between incubation with sHyal2 and the low-pH pulse, but not before the sHyal2 incubation (Fig. 1F). To test if low pH could modulate the requirement of sHyal2 for fusion, we preincubated 293 cells with different amounts of sHyal2, followed by treatment with different low pH conditions for 1 min. Although 1 µg/ml of sHyal2 induced visible syncytia at pH 4.0, 5 µg of sHyal2 was necessary to trigger discernable fusion at pH 5.0; even 10 µg or more of sHyal2 barely triggered fusion at pH 6.0 (pictures not shown). Collectively, these results demonstrate that sHyal2 functions in cooperation with low pH treatment to trigger JSRV Env-mediated fusion, likely by
inducing one or more fusion intermediates that are sensitive to the binding of JSRV C-HR2 peptide.

**Severe truncation of the cytoplasmic tail of JSRV Env at the N-terminus results in syncytia formation in 293 cells at neutral pH**

In a previous study we showed that severe truncation of the cytoplasmic tail (CT) of JSRV Env increases the fusion activity of Env, and even induces membrane fusion at neutral pH in Hyal2-overexpressing 293 cells [431]. Interestingly, we found that the fusion activity of these mutants at neutral pH was still significantly enhanced by a subsequent low-pH pulse [431]. Here we asked whether severe truncation of JSRV Env CT also relieves the requirement of receptor overexpression for fusion induction. Indeed, we found that all three severe truncation mutants, CT589 and CT572 (18 and 1 aa remaining in the CT, respectively, Fig. 2A), and Δ572-589 (N-terminal 18 aa of CT was deleted, Fig. 2A), induced small but reproducible syncytia at neutral pH in 293 cells expressing endogenous levels of Hyal2 (Fig. 2B). Intriguingly, the fusion activities of these mutants at neutral pH could not be further enhanced by a 5-min pulse with pH 5.0, unless the cells were preincubated with sHyal2 (Fig. 2B). Although CT572 and Δ572-589 induced more syncytia at neutral pH than did CT589, the fusion activities of CT572 and Δ572-589 at low pH were relatively low, and even lower than that of wildtype (Fig. 2B), likely due to their reduced surface expression [431], or/and prematured fusion activation. The generally increased fusogenicities of these truncation mutants correlated with their enhanced SU shedding into culture media relative to that of wildtype [[431], and data not shown]. Overall, these data demonstrate that, while severe truncation of the CT of JSRV Env
alleviates the thresholds of both low pH and receptor overexpression for fusion, full activation of their fusogenicity still require stepwise triggers of both Hyal2 and low pH.

**sHyal2 promotes shedding of JSRV SU in cells expressing JSRV Env**

SU shedding is regarded as an important, though not necessary, feature of retrovirus fusion and infection [418]. To address possible effects of sHyal2 on JSRV SU shedding, Env-expressing 293 cells and 293/LH2SN cells, the latter of which overexpress human Hyal2 approximately by 10-fold [431], were pulse-chase labeled in the presence of different amounts of sHyal2. While sHyal2 had no effect on JSRV Env expression and processing over the 3-h chase period (Fig. 3A), as would be expected, pronounced shedding of SU into the culture medium was observed in 293 cells treated with sHyal2 in a dose-dependent manner (Fig. 3B). The basal level of JSRV SU shedding in 293/LH2SN cells appeared to be slightly higher than in 293 cells, yet no apparent increase after treatment with sHyal2 was observed in 293/LH2SN cells (Fig. 3B). Of note, 293/LH2SN cells exhibited markedly less Env expression particularly the mature SU and TM, than 293 cells, likely due to proteasomal degradation of Env by overexpressed Hyal2 in 293/LH2SN cells. In support of this possibility, treatment of the Env-expressing 293/LH2SN cells with MG-132, a proteasomal inhibitor, drastically increased Env levels in the cells (data not shown). Collectively, these data demonstrate that sHyal2 promotes JSRV SU shedding in Env-expressing 293 cells, and suggest that sHyal2 causes conformational changes in SU on the cell surface, resulting in a weakened association between SU and TM (see discussion).
sHyal2 induces conformational refolding of TM in JSRV Env-pseudotyped retroviral particles

To gain biochemical evidence for the role of Hyal2 in JSRV Env-mediated fusion activation, we developed a TM oligomerization assay and assessed possible conformational changes of JSRV TM induced by sHyal2, low pH, or both. In the absence of the crosslinker, DSP, JSRV TM exists predominantly as a monomer of ~37-kDa, although a minor species of ~90-kDa, likely the SU-TM heterodimer, was also detected in all samples (Fig. 4A). In the presence of DSP, a ~120-kDa species was apparently induced by low pH (lane 6, Fig. 4A), and its intensity was substantially enhanced by preincubation with sHyal2 (lane 8, Fig. 4A). Interestingly, sHyal2 alone caused a slight increase in formation of the ~120-kDa species (lane 7, Fig. 4A), yet appeared to enhance the 37-kDa monomers out of the high-molecular-weight (HMW) species (compare lane 5 and 7, Fig. 4A). This latter observation was further confirmed by an experiment showing that sHyal2 increased the proportions of TM monomers but reduced HMW species and SU-TM heterodimers in a dose-dependent manner (Fig. 4B). Together, these results imply that sHyal2 may disrupt the SU-TM associations of JSRV Env in the pseudovirions, similar to the observation in Env-expressing 293 cells (Fig. 3), although we were unable to detect JSRV SU by Western blot in the oligomerization assay.

sHyal2 modulates the threshold of low-pH or heat required for TM oligomerization

We next tested if sHyal2 could modulate the thresholds of low pH and/or high temperature required for TM oligomerization. In the absence of sHyal2 the pH threshold for inducing the ~120-kDa species was ~pH 5.5, but it shifted to ~pH 6.5 in the presence of 1.5 µg of sHyal2 (Fig. 4C). These pH thresholds appeared to be higher than those observed
in the fusion assays [433], possibly because that the biochemical oligomerization assay is more sensitive than the phenotype-based fusion assay. Similarly, the temperature threshold for detecting the ~120-kDa species was reduced from 65°C to 50°C in the presence of sHyal2 (Fig. 4D). Taken together, these data demonstrate that sHyal2 effectively reduces the energy barriers that must be overcome by low pH or heat in order to form the ~120-kDa species. Our results also show that the JSRV Env protein in the mature MoMLV pseudoparticles is metastable, similar to that of influenza HA virus and ASLV Env [205, 434-436].

**Stability of JSRV TM oligomers induced by sHyal2 and low pH**

The stability of JSRV TM oligomers, in particular the ~120-kDa species, which was specifically induced by low pH and/or sHyal2, was also assessed. TM oligomers induced by a combination of low pH and sHyal2 were stable in urea, up to 2 M; whereas sHyal2-induced oligomers were relatively unstable, and disassociated even with 0.5 M urea (Fig. 5A). Interestingly, the ~120-kDa species, induced either by low pH plus sHyal2 or by sHyal2 alone, were sensitive to SDS treatment, even at very low concentrations (0.01% or higher) (Fig. 5B). These results are in sharp contrast to those reported for ASLV, whose 6-HBs and other HMW species are virtually resistant to 1~2% SDS even without crosslinking [205, 422]. However, our results are similar to those of MoMLV, whose TM oligomers are also less stable in SDS [437].
sHyal2 enables JSRV Env pseudovirions to transduce non-permissive cells, but blocks their entry into permissive cells

The apparent role of sHyal2 in JSRV Env-mediated fusion prompted us to further test whether sHyal2 can induce JSRV vector transduction of non-permissive cells. To this end, JSRV Env pseudovirions were allowed to bind to non-permissive HeLa cells at 4°C for 2 h, and the virion-cell complexes were incubated with sHyal2 for 48 h before the cells were analyzed for infectivity by flow cytometry. As shown in Fig. 6A, sHyal2 enabled transduction of HeLa cells by JSRV pseudovirions, albeit with low efficiency, but had no effect on those of VSV. In contrast, sHyal2 markedly reduced the transduction efficiency of JSRV-permissive HTX cells, which were prebound with JSRV pseudovirion particles, in a dose-dependent manner (gray bars, Fig. 6B). As would be expected, preincubation of JSRV pseudovirions with sHyal2 (1.5 µg/ml) before binding to HTX cells, virtually blocked vector transduction (black bars, Fig. 6B). These results strongly argue that, in addition to blocking virus binding to target cells, sHyal2 also inactivates JSRV Env pseudovirion infectivity, possibly by triggering premature conformational changes of TM, as shown in Fig. 4. The potent effect of sHyal2 on the inactivation of JSRV Env pseudovirions was in sharp contrast to that of low pH, which has no significant effect [431]. The results of HTX cells likely explain, at least in part, why sHyal2-mediated transduction of a JSRV vector into non-permissive HeLa cells was inefficient and not linearly dose-dependent (Fig. 6A).
4.6 Discussion

JSRV is a unique retrovirus in that its native Env functions as an active oncogene in addition to mediating cell entry. However, unlike most retroviruses that are pH-independent, JSRV entry requires dynamin-dependent endocytosis and a low pH [431, 432]. Interestingly, we recently observed that membrane fusion mediated by JSRV Env in vitro could only be induced by low pH in cells that overexpress functional Hyal2 [431], suggesting that Hyal2 may play an active role in the fusion activation process. Using a soluble form of the JSRV receptor, sHyal2, we have demonstrated here that low pH is indeed not sufficient to induce fusion by JSRV Env, but that receptor binding is also required for this process (Fig. 1). We conclude that, similar to ASLV, oncogenic JSRV has borrowed features of both pH-independent and dependent viruses for its fusion activation and entry, which involves stepwise and cooperative triggers from both the receptor and low pH.

However, there are some striking differences between JSRV and ASLV in terms of fusion activation by receptor binding or priming. First, we show that sHyal2 induces pronounced SU shedding of JSRV Env in 293 cells at neutral pH (Fig. 3), and this is in sharp contrast to that of ASLV, where soluble Tva does not cause apparent disassociation between SU and TM [438] (see detailed discussion below). Second, we find that the ~120 kD species, likely the 6-HB, or pre-6-HB intermediate, of JSRV Env, is much less stable in SDS (Fig. 5A), in contrary to the counterpart of ASLV Env, which is resistant to SDS up to 2% [205, 422]. This could be due to the relatively short lengths of N- and C-HRs of JSRV Env as compared to those of ASLV[439], and/or to their intrinsic differences in specific amino-acid interactions, or technical limitations (for example, SDS may affect the effect of
crosslinking by DSP). The relative low stability of JSRV TM oligomers also explains, at least partly, why crosslinking is required for detection of JSRV TM oligomerization (Fig. 4). Third, sHyal2 potently inactivates the infectivity of JSRV Env pseudovirions in a step(s) after virus binding (Fig. 6B), consistent with the observation that sHyal2 induces the TM conformation refolding of JSRV Env at neutral pH (Fig. 4A). This feature appears also to be different from that of ASLV, in which both soluble Tva and low pH are required to inactivate the viral infectivity [434]. In addition, the fusion peptide of ASLV is quite different from that of JSRV and of most retrovirus Envs, which may contribute to its unusual stability of TM oligomers, including pre-6-HB intermediate [201]. For example, the ASLV fusion peptide is internal, and is located near the N-terminus of its TM, with two flanked cysteines and a central proline to be critical for fusion activation [440, 441]. By contrast, the putative fusion peptide of JSRV Env is located at the immediate N-terminus of TM, which is generated via SU/TM cleavage and does not contain these cysteines and proline residues. Collectively, the mechanism of action by which JSRV receptor, Hyal2, primes Env to trigger fusion activation is quite different from that of ASLV, and JSRV likely provides an alternate valuable model for study of membrane fusion and cell entry by pH-dependent viruses.

We propose the following model for JSRV Env-mediated fusion activation. Upon receptor binding, JSRV SU undergoes conformational changes that cause it to disassociate from TM. This would relieve its intrinsic restriction on TM, resulting in exposure of the fusion peptide and its subsequent insertion into the plasma membrane. Although our current study did not directly address the conformational changes of SU and the association of TM with the cell membrane, it was evident that sHyal2 promotes pronounced JSRV SU
shedding in Env-expressing cells (Fig. 3), and also possibly in Env pseudovirions as well (Fig. 4A and B). In addition, we observe that JSRV SU/TM heterodimer can be disassociated by reducing agents in the protein sample buffers, suggesting that they are indeed linked by disulfide bond (data not shown), as that of most retroviruses. It is noticeable that JSRV SU contains a CXXC motif, a conserved amino acid sequence that has been previously shown to be important for fusion activation in other retroviruses by mediating the switch from an intersubunit (SU-TM) to an intra-SU subunit disulfide bond [162]. By contrast, ASLV does not contain the CXXC motif, but its receptor has been recently shown to induce the formation of reactive thiolates in SU, which are essential for fusion and infection, independent of isomerisation of the SU-TM disulfide bond [438]. Whether or not the CXXC motif of JSRV SU, and/or the potential formation of receptor-induced thiolates, mediates JSRV SU shedding and fusion activation remains to be investigated.

Once the fusion peptide inserts into the plasma membrane, the TM of JSRV Env may become elongated and form one or more pre-hairpin intermediates that are sensitive to JSRV C-HR2 peptide binding. At this point, low pH is necessary to trigger dramatic TM conformational refolding to form 6-HB and for subsequent fusion. We found that although the JSRV C-HR2 peptide effectively inhibited JSRV Env-mediated membrane fusion (Fig. 1F) and infection (data not shown), we have been unable to block formation of the ~120-kDa species, suspected to be the 6-HB of JSRV TM, using the JSRV specific C-HR2, even at extremely high concentrations (up to 2 mg/ml). It is possible that the ~120-kDa form is a heterogeneous mix, containing both pre-6-HB intermediates and stable 6-HBs; alternatively, this form is a precursor of JSRV 6-HB, with a true 6-HB form remaining to
be identified. Interestingly, the ASLV C-HR2 peptide, R99, does not always block formation of 6-HB species, yet potently inhibits ASLV Env-mediated fusion and infection [205, 439, 442]. It should be added that all JSRV Env pseudovirions used in this study were produced from 293T or 293 cells (due to their high transfection efficiency), and it is possible that the endogenous Hyal2 expressed in these cells have prematurely activated JSRV Env in the viral particles, which may contributed to formation of the ~120-kDa species by low pH alone, even without sHyal2 preincubation (Fig. 4B). Work is underway to address this issue, and further characterize the JSRV fusion intermediate(s) and the formation of its 6-HBs.

Viruses have evolved different strategies to control fusogenicity to both avoid extreme cell-cell fusion in producer cells, and to ensure that fusion activation occurs in the right place at the right time of target cells. For pH-independent retroviruses, particularly gamma- and beta-retroviruses, control of fusogenicity is largely achieved by the R peptide, a stretch of ~16 aa present at the C-terminus of Env cytoplasmic tails, which inherently inhibits Env fusogenicity but is cleaved on virus budding [443-446]. For pH-dependent viruses, including retroviruses, fusogenicity is controlled by low pH or by low-pH-dependent cellular protease activities of target cells [402, 403, 405, 408, 447-449]. Whether or not JSRV Env contains an R peptide is currently unknown, however, it is clear from this and our previous studies that its fusogenicity is controlled by both receptor binding and low pH, and is modulated by its relatively long CT, in particular that of the N-but not the C-terminus, of CT [431]. It is interesting that the oncogenic domain of JSRV Env is also primarily located at its N-terminus and central region [450], and that severe truncation of the JSRV Env CT at the N-terminus resulted in a loss of cell-transforming activity, but enhanced the Env fusogenicity at low pH [431], and even rendered small but
reproducible syncytia formation at neutral pH in normally fusion-inefficient 293 cells (Fig. 2). Therefore, JSRV appears to have evolved a more complex mechanism in order to tightly control its fusogenicity in transformed cells, which may benefit its oncogenic potential and replicative fitness. Indeed we have not observed syncytia formation in any type of JSRV Env-transformed cells, despite extraordinarily high levels of Env expression in these cells and their much acidified culture media (unpublished observations). This could be due to the absence of Hyal2 expression (in mouse NIH 3T3 or rat 208F cells) [386], or because the Hyal2 receptor is severely downregulated by JSRV Env (in canine MDCK cells) [451]. Convincingly, incubation of Env-transformed cells with sHyal2 induced obvious syncytia formation at low pH, though with different efficiencies depending on the cell type (data not shown). Whether or not the mechanism of fusion activation by JSRV Env and its unique mode of regulation by the CT also operate in productive JSRV infection should be investigated in future studies.

### 4.6 Acknowledgements

We thank Vlad Vigdorovich and Dusty Miller for the generous gift of the S2 cell line stably expressing sHyal2. This work was supported by the Canadian Institutes of Health Research (CIHR) to S.-L.L. M.C. was supported by scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC). S.-L. Liu is a Canada Research Chair in Virology and Gene Therapy.
Fig. 4.1. sHyal2 mediates syncytia formation of JSRV Env in 293 cells at low pH. (A) Purification of sHyal2. sHyal2 (~50 kDa) was purified using Ni-NTA columns, with purity determined by SDS-PAGE and Coomassie blue staining. (B) sHyal2 specifically binds to 293 cells expressing a FLAG-tagged JSRV Env, whose expression was confirmed by an anti-FLAG antibody. (C) Syncytia formation by JSRV Env in 293 cells (expressing GFP) is induced only by sHyal2 plus pH 5.0, but not by either one alone. (D) A JSRV Env chimera, containing the ENTV SU and with reduced binding to Hyal2, requires an increased concentration of sHyal2 for fusion. (E) sHyal2 acts before but not after the pH 5.0 pulse for fusion induction. These experiments employed 5 µg sHyal2. (F) JSRV C-HR2 peptide acts after sHyal2 incubation and before low pH pulse to inhibit fusion. The assays employed 5 µg of sHyal2 and 30 µg of C-HR2 peptide.
Fig. 4.2. Severe truncation of the cytoplasmic tail (CT) of JSRV Env results in syncytia formation at neutral pH in 293 cells. (A) CT sequences of JSRV Env wildtype (WT, JS7 strain, GenBank AF357971) and truncations. MSD: membrane-spanning domain. (B) Syncytia induction. Env-expressing 293 cells were treated with or without 5 μg of sHyal2, followed by a pulse with pH 7.4 or pH 5.0 buffer for 5 min. Syncytia formation was determined by fluorescence microscopy. The numbers of syncytia per field at neutral pH were scored and averaged, as shown inside the pictures. Arrows indicated fused cells.
A

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B

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WT

0.2 ± 0.1

CT589

2.0 ± 0.8

CT572

2.8 ± 0.7

Δ572-589

3.9 ± 1.0
Fig. 4.3. sHyal2 promotes shedding of JSRV SU. 293 or 293/LH2SN cells expressing JSRV Env were pulse-chase labelled in the presence or absence of sHyal2; cell lysates and culture media were harvested, and immunoprecipitated, respectively, using anti-FLAG beads. Samples were resolved by SDS-PAGE and analyzed by autoradiography. (A) Env expression and processing in cell lysates. (B) JSRV SU shedding into culture media. Env: JSRV Env precursor; 293/LH2SN: 293 cells overexpressing human Hyal2.
A

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Env →
SU →
TM →

B

Shed SU →
**Fig. 4.4. JSRV TM oligomerization induced by sHyal2 and low pH.** (A) Purified JSRV Env pseudovirions were incubated with or without 1.5 μg of sHyal2, followed by a treatment with pH 7.4 or pH 5.0 buffer. Samples were crosslinked by 25 μM DSP, or were left untreated, and TM oligomerization was analyzed by Western blot (WB) using an anti-FLAG antibody. (B) Effect of sHyal2 on TM oligomerization. Purified virions were incubated with indicated amounts of sHyal2, or were treated with neutral or a low pH, which served as negative and positive controls, respectively. Samples were crosslinked and analyzed by WB. (C, D) sHyal2 modulates the thresholds of low pH (C) and temperature (D) required for JSRV TM oligomerization. Pseudovirions, preincubated with 1.5 μg of sHyal2 or an equal volume of PBS, were treated with indicated pH conditions (C) or temperatures (D) for 5 min, followed by crosslinking with DSP and analyzed by WB.
Fig. 4.5. Stability of TM oligomers induced by sHyal2 and low pH. JSRV Env pseudovirions, preincubated with 1.5 µg of sHyal2 alone or 1.5 µg of sHyal2 plus low pH, were subjected to indicated concentrations of urea (A) or SDS (B) for 5 min at 37°C before being crosslinked by DSP and analyzed by WB.
Fig. 4.6. sHyal2 enables transduction by JSRV Env pseudovirions of non-permissive cells (A), but blocks their entry into permissive cells (B). (A) HeLa cells were bound by JSRV Env or VSV-G pseudovirions encoding GFP by spinoculation, followed by incubation with indicated amounts of sHyal2. The transduction efficiency was analyzed by flow cytometry 48 h post-infection. Representative dot plots are shown, with % GFP+ cells indicated. The fold changes from four independent experiments were averaged and plotted. (B) HTX cells were infected with JSRV Env or VSV-G pseudovirions that were preincubated (before binding) with indicated amounts of sHyal2, or were bound first with pseudovirions and then incubated with indicated amounts of sHyal2 (after binding). In both cases, unbound sHyal2 and virions were removed from cells by washing, and viral infectivity was assessed by flow cytometry 48 h post-infection.
CHAPTER 5

GENERAL DISCUSSION
Discussion

Throughout the chapters, we have learned more about JSRV and ENTV Env-mediated fusion. We found, in chapter 2, that JSRV is a pH-dependent virus that requires a low pH for entry and fusion. Using a similar approach, we demonstrated in chapter 3 that ENTV Env necessitates an even lower pH than JSRV for fusion activation. In chapter 4, we uncovered that receptor binding is an essential step to prime the envelope sensitive to low pH treatment. In addition, throughout the thesis we found that the Env protein is tightly modulated by several different mechanisms, such as the inhibition by the cytoplasmic tail, the modulation of the pH requirement by the TM subunit, and the modulation of the fusion activity by the SU subunit. As detailed discussion has been provided at the end of each chapter, in this general discussion we will focus on the general findings as a whole and put them in context with other viral fusion proteins. In addition, we will use our knowledge on other viruses to gain possible perspectives of what remains to be determined and discuss some of the future directions.

Mechanism of JSRV Env fusion

Our data point out to a complex fusion activation mechanism of JSRV Env as illustrated in figure 5.1. In this model, the SU subunit is not only an important restriction factor on TM activation, but is also critical in modulating the activation of the protein. Importantly, low pH is the key for the triggering of the ultimate conformation of TM leading to fusion. Thus, the activation mechanism of JSRV Env is similar to that of ASLV Env, but exhibits some important differences. As expected, JSRV Env also shares some important similarities with other retroviral envelopes in terms of the position of the fusion
peptide, CXXC motifs, and the proline-rich region in the SU subunit, which are important features of retroviral envelope proteins.

**Figure 5.1 Proposed model for the activation of JSRV Env-mediated fusion.**

Based on HA crystallographic data, in the postfusion conformation, JSRV Env TM is folded with a C-terminal core, shown in orange, and is locked by its interaction with the SU subunit. For clarity, only a second monomer of the trimer is shown in gray. Upon binding of Hyal2 with SU, the latter subunit is shed and, consequently, the clamp on TM is released. This results in a first conformational rearrangement where TM adopts a rod-like structure; thereby inserting its fusion peptide, shown in purple, into the outer leaflet of the cell membrane which is possibly that of the endosome. The acidic environment of the endosome’s lumen triggers another change in conformation. TM then refolds into a hairpin structure through interaction between the C- and N-terminal heptad repeats eventually leading to fusion of the cellular and viral membrane. Note the position of the N-terminal heptad repeat, labelled in yellow, at the post-fusion conformation which is found inside the trimer. In addition, as suggested by the study in chapter 2, the cytoplasmic tail, shown in green, inhibits fusion activation and possibly interacts with the membrane [322].
First trigger: SU binding.

Our data reveal that receptor interaction is absolutely required for fusion. This conclusion is based on the need for Hyal2 overexpression to observe cell fusion. In addition, non permissive cells can be infected by JSRV if the receptor is provided in trans. Another interesting clue is the modulation of fusion activity by the binding affinity of SU for the receptor, indicated by the reduced fusogenecity of all constructs bearing the low binding affinity ENTV SU as compared to those harbouring JSRV SU.

Based on our results, we propose that upon Hyal2 binding, the SU-TM interaction is altered, resulting in the shedding of SU from the complex. Although, whether or not SU completely dissociates from its complex with TM in the viral particles remains to be determined. For instance, during the trigger of HA, the surface subunit HA1 is only displaced but still interacts with the transmembrane fusogenic subunit HA2 [105]. In the case of HIV, the release of gp120 does not always correlate with subsequent fusion, but is still observed during fusion [176]. Therefore, this feature strongly resembles the HIV Env fusion activation and also that of MLV Env. As explained in the introduction, MLV Env SU contains an isomerase activity provided by its CXXC motif [162]. It also possesses a proline-rich region important to transmit the signal from the receptor binding domain (RBD) to the CXXC motif. With the involvement of a CX6C motif in TM of MLV Env, the two subunits are linked by an intersubunit disulfide bond, which is rearranged into an intrasubunit disulfide bond by isomerisation. Interestingly, JSRV SU also contains a proline-rich region downstream of the RBD, and it is followed by the CXXC motif. However, the conserved CX6C motif is slightly different in JSRV TM and possesses a CX7C motif instead. It is still unknown if JSRV SU and TM are bound by disulfide linkage. However, we found that the sensitivity of SU-TM interaction to a reductive agent,
β-mercaptoethanol, is cell-type dependent and appears to correlate with Hyal2 status (data not shown). On the other hand, HIV Env gp120 (SU) and gp41 (TM) are not linked by a disulfide bond, yet remain bound together through a non-covalent interaction. In this case again, upon receptor binding, SU undergoes a change in conformation. We still do not know if the Hyal2 interaction causes a change in conformation of SU, and future experiments aim to address this issue. This is potentially important because it could provide an answer as to how Hyal2 causes dissociation or displacement of JSRV Env SU and the first important conformational change. In contrast, ALSV Env SU and TM are linked by a disulfide bond that cannot undergo isomerisation since it does not contain a CXXC motif. Consequently, SU shedding is unlikely to happen, and, in fact, has never been reported. Thus, the effect of ALSV SU following receptor interaction most probably resembles the initial effect of low pH on influenza HA, whereby HA1 is displaced, but not shed.

SU-Hyal2 interaction results in conformational change in JSRV TM, rendering the latter sensitive to a C-terminal heptad repeat peptide (HR). Inhibitory HR peptides are thought to act by binding to the opposite heptad repeat region, thus competing with the internal analogous HR for the formation of the hairpin. Therefore, this conformation is probably the rod-like structure. For ASLV Env, it was shown that receptor binding leads to insertion of the fusion peptide into the target membrane which was demonstrated by liposome association [85]. To test whether this was the case for JSRV, we have attempted to observe liposome association by coflotation assay after Hyal2 binding, but were unable to detect lipid interaction so far (data not shown). However, as mentioned previously, the fusion peptide of ASLV is distinct from other class I fusion proteins; and moreover, it is an internal fusion loop, which was shown to surprisingly insert itself deeper in the lipid leaflet, as compared to other N-terminal fusion peptides [97]. Thus, it is possible that the N-
terminal fusion peptide of JSRV Env does not insert itself deeply enough for the stable association to take place. Another possibility is that the post-Hyal2 binding conformation of TM is not stable. We have shown in chapter 4 that sHyal2 can allow infection of JSRV in nonpermissive cells, yet reduces transduction of permissive cells, suggesting that Hyal2 inactivates the virus possibly by the premature conformational rearrangement. In other words, the intermediate between the Hyal2 binding step and the step that requires the low pH is potentially unstable, and may refold into an inactive conformation. This intermediate is likely the rod-like structure with the fusion peptide inserted in the target membrane, but this assumption still requires further investigation.

**Second trigger: low pH**

Following receptor binding, a low pH trigger is required for the completion of fusion. This necessity was demonstrated by syncytium induction and cell-cell fusion assays for JSRV and ENTV in chapters 2 and 3, respectively. This prerequisite also applies for infection, which can be inhibited by the constant presence of lysosomotropic agents or the irreversible proton pump inhibitor Bafilomycin A1. In addition, we showed that increasing the amount of sHyal2 cannot bypass the need for the low pH trigger.

The oligomerisation assay presented in chapter 4 shows that low pH triggers the formation of high molecular weight species of TM. It is very likely that acidic conditions have a direct effect on the extracellular region of TM, since we showed that the region responsible for the differential pH requirement between JSRV and ENTV Env is located in their TM subunits. As described in the introduction, histidines are regarded as pH sensors, and it is possible that they play a role in the pH dependency of JSRV and ENTV Env. Interestingly enough, ENTV and JSRV Env both contain 10 conserved histidine residues in
the TM ectodomain. While these conserved histidines do not explain the difference in pH requirement, they may be important in the modulation of fusion. Investigation of the fusion activity of histidine mutants will be very helpful in assessing their importance.

Because the JSRV fusion protein is mostly sensitive to the HR peptide before but not during of after low pH treatment, we suspect that acidic conditions are needed to proceed from the rod-like structure to the hairpin. The low pH trigger causes a final conformational rearrangement in the TM subunit characterized by the formation of relatively stable trimers as detected using our oligomerisation assay. However, while the oligomers are stable under high concentration of urea, they are highly sensitive to SDS treatment. The stability of the post-fusion conformation is a major difference between JSRV and ASLV Env. ASLV has been shown to form oligomers in its postfusion conformation that are resistant to 2% SDS treatment [205]. In the case of JSRV Env, a cross-linker is required to observe high molecular weight species, suggesting that the six-helix bundle is not as stable as that of ASLV. As mentioned earlier, the final conformation, the six-helix bundle, is formed by the interaction between the N- and the C-terminal heptad repeats (HR). Similar as some SNAREs, the association has been shown to be relatively resistant to SDS treatment. Based on sequence analysis, the predicted HRs of JSRV are shorter than those of ASLV [239]. Thus, it is possible that because the interface of interaction is shorter, the association of the interacting N- and C-HRs is weaker. We are currently analysing the interaction between the two heptad repeat regions of JSRV and ENT, which will likely provide an answer to the relative instability of the six-helix bundle.
Regulation of JSRV and ENTV fusion activation

In the early stages of this project, we were driven by the idea that there must be a regulatory mechanism controlling JSRV Env fusion activity, otherwise it would lead to fusion of the infected transformed cells with the receptor expressing-neighbouring cells, thereby detrimental for viral fitness. The first regulatory mechanism we identified is the requirement of a low pH, as discussed in details above. However, other mechanisms may also exist, since the lung environment of the transformed cells is acidic [362]. In chapter 2, we described one regulatory mechanism used by several retroviruses, which is modulation of the fusion activity by the cytoplasmic tail. However, in contrast to the retroviral envelopes that contain a C-terminal R peptide, JSRV Env CT regulatory region is located within its N-terminus. The study in chapter 3 revealed that the TM subunit contains elements that dictate the differential pH requirement of JSRV and ENTV. Finally, our study demonstrated that the SU-Hyal2 interaction modulates the fusion activity. Taken together, our studies reveal that JSRV Env fusion activity is tightly regulated, which might be important for viral oncogenesis and fitness.

CT regulates the fusion activity

In chapter 2, we showed that JSRV Env cytoplasmic tail negatively regulates its fusion activity. The mechanism by which it restricts the envelope protein is still unclear. However, it seems that the SU-TM interaction is modified, as SU shedding was observed. Inside-out signalling has been shown for other viruses [359]. It is hypothesized that a modification in the cytoplasmic tail can result in alterations in the extracellular portion. In the case of JSRV Env, it is possible that truncation of the cytoplasmic tail changes the
ectodomain of TM in a way that its interaction with SU is weakened. We also observed a similar effect of CT truncation on ENTV Env fusion, whereby the envelope protein with a single amino acid in its CT exhibits a significantly higher fusion activity (data not shown). Based on the clamp-release mode of fusion activation, the interaction with SU restricts TM to refold. Consequently, alteration of the SU-TM association could theoretically modulate the fusion activity of the protein. This can partly explain the increased fusogenicity and the fusion at neutral pH. One interesting observation was that fusion at neutral pH could also be observed for the truncation mutants in 293 cells expressing endogenous levels of Hyal2. This suggested that not only could the truncation be activated at neutral pH, but also that their activation was less dependent on receptor binding. However, as demonstrated in chapter 2, the pseudovirions harbouring JSRV truncation still require acidic vesicles for infection and robust syncytia necessitates the receptor and low pH (see chapter 4). Therefore, it is difficult to point out which step is alleviated by the CT truncation mutants. One possibility is that the metastable state of the truncations is a relatively unstable higher energy state compared to that of the WT. Thus, it is possible that the Env would refold spontaneously into the low energy post-fusion conformation without the need of strong triggering by receptor binding and acidic pH. This could also explain the relatively low number of fusion events in these conditions since this triggering is not synchronised with the other Env. Indeed, it is very likely that pore formation requires the concerted action of more than one Env. Further experiments, using the fusion assay, will be aimed at determining the amount of energy and/or temperature required by the truncation mutants to form the post-fusion conformation compared to the WT.
One remarkable observation is that, in contrast to the common R-peptide restriction of Env fusion, only the N-terminal region of JSRV Env CT has an inhibitory effect on fusion. This was shown in chapter 2 for N-terminal truncation as well as in chapter 4 with the N-terminal deletion JSRV Env d572-589. Interestingly, the N-terminal residues of JSRV cytoplasmic tail, from 572 to 589, are predicted to form coiled-coils that may interact with the membrane [322]. Thus, it is possible that the N-terminal region serves to stabilise the Env, either by forming coiled-coils with the other monomers of the trimer or by interacting with the membrane, which likely explain the truncation instability in normal conditions and fusion at neutral pH.

It has been shown that changes in cytoplasmic structures can affect the conformation of the ectodomain of TM [360, 452]. Consequently, it is likely that the N-terminal truncation of JSRV Env causes a change in the extracellular domain of TM, and this would affect the SU-TM interaction. However, the conformational change of TM by cytoplasmic tail truncation remains to be determined. While it is still unclear if JSRV or ENTV Env contains an R-peptide, our data suggest that the deletion of the 16 C-terminal residues does not significantly affect the fusion activity of the envelope, suggesting that the involvement of R-peptide as a regulatory mechanism is unlikely.

**TM as a sensor of pH**

Based on our model, low pH mainly has an effect on TM refolding from a rod-like structure to the hairpin forming the six-helix bundle. As a result, it is expected that TM is the subunit that is directly affected by the pH. As a matter of fact, in chapter 3, we observed that the TM subunit is responsible of ENTV Env very low pH requirement. It is possible that histidine residues become protonated when the virus is found in acidic compartments.
Interestingly, whereas the histidine residues in the ectodomain of JSRV and ENTV Env are conserved, other residues close to those histidines may cause an alteration of the pKa of the latter.

Mutations in the HA2 ectodomain have also been shown to change the pH optimum for fusion. Those could be found in the fusion peptide, the heptad repeat regions, the interface between the HA1 and HA2 subunits and regions of HA1 or HA2 surrounding the fusion peptide in the native conformation [453]. Consequently, the pH requirement can be modulated by alteration of the initial conformation through modification of the interaction of the two subunits or internal changes close to the fusion peptide, which possibly modifies the stability of the pre-fusion state. Another possibility is the modulation of intermediates of fusion with the contribution of residues within the fusion peptide potentially affecting the rod-like structure step. Finally, residues in the coiled-coil regions or HR as well as the fusion peptide can play a role in the modulation of the pH requirement by the alteration of the post-fusion step or six-helix bundle. There are only six residues that are different in the ectodomain of JSRV and ENTV Env. None of them are located in the putative fusion peptide, but there is one substitution from a leucine to a valine residue found in the C-terminal heptad repeat region. Since no crystal structures of JSRV or ENTV Env ectodomains are currently available, it is not possible to know if some of the different residues are located at the SU-TM interface or the fusion peptide pocket. We are currently performing mutational analysis to identify which amino acid substitution(s) is/are responsible for the differential pH requirement of JSRV and ENTV Env. In addition, the membrane spanning domain and the cytoplasmic tail are the most divergent region of the TM of ENTV and JSRV Env. It is possible that these regions contribute to the differential
pH requirement. The generation of further chimeras of ENTV and JSRV MSD and CT will be used to clarify this issue.

**Modulation of fusion by SU-Hyal2 interaction**

In chapter 3, we showed that ENTV exhibits lower binding to human Hyal2 than JSRV. This is reflected by its very low titer in 293 cells expressing endogenous levels of Hyal2 as compared to that of JSRV vector transduction. Overexpression of the receptor leads to a $10^5$-fold increase in ENTV titer, while the JSRV titer is enhanced by $10^2$-fold only. There was one report suggesting that ENTV’s low titer could be due to the requirement for an unknown co-receptor [299]. Our data indicate that at least for HTX cells and 293 cells, the main determinant is the level of expression of the receptor. While the affinity of soluble SU subunits of ENTV and JSRV for Hyal2 have been compared [283], supporting a model that the SU of ENTV Env possesses a low binding affinity for the receptor compared to JSRV, a possible cooperative binding of ENTV SU to Hyal2 as well as the determinants in SU that regulate this binding difference, have never been investigated. In addition, this low binding affinity of ENTV SU to the receptor explains its low fusogenicity compare to that of JSRV Env, suggesting that ENTV Env fusion activation mechanism is similar to that of JSRV, where both receptor binding and low pH play active roles in the fusion process.

Based on sequence comparison and mobility on gel shown in chapter 3, ENTV SU contains additional glycosylation sites compared to JSRV SU. As discussed in the introduction, the level and type of glycosylation can be critical for receptor interaction. Therefore, it is possible that the glycosylation of ENTV SU is responsible for the low binding to human Hyal2. However, it is unclear whether these extra glycosylations occur in
its natural host, or whether it provides a better binding to ovine as compared to human Hyal2. In addition, glycosylation has also been shown to be important for evasion of antibody neutralisation and for protease resistance. While an antibody-mediated response is unlikely, since there is almost no immune response to JSRV or ENTV infection in sheep and goat, it is possible that these extra glycosylations confer protection from the very acidic environment or protease activity found in the fusion compartment. This could be even more important for ENTV, because of its requirement for a more acidic pH for fusion. Investigation of the residues that are different in ENTV and JSRV receptor binding domain of SU will likely provide more information on the determinants responsible for the interaction with the receptor.

**JSRV and ENTV entry pathways**

After viral binding to the cell surface via Hyal2, the virus is internalized. The endocytic pathway used by JSRV is still unclear, but appears to require dynamin [364]. In addition, because Hyal2 is a GPI-anchored protein and is probably localised in the lipid rafts, it is possible that it uses the caveolar-endocytic pathway. It is unknown if the initial binding to Hyal2 at the surface of the host cell activates the protein and remains to be determined. For instance, the recent study on HIV reveals that receptor interaction at the surface of the cell induces hemifusion, yet complete fusion of the viral membrane with that of the cell occurs in the endosome. In the case of JSRV and ENTV, hemifusion could occur rapidly at the surface of the cell upon receptor binding; however, as shown by the syncytium assay, the endogenous level of the receptor in 293 cells is insufficient to induce cell fusion. Thus, it is possible that a high density of receptor is required to activate the JSRV and ENTV Env protein. Interestingly, hyaluronidases are lysosomal proteins, and
while it remains to be determined, it is possible that Hyal2 is found in a high concentration in acidic vesicles in addition to the cell surface. One hypothesis is that the initial JSRV binding to Hyal2 leads to internalization of the virus but not activation of the fusion process. Inside acidic compartments, the virus could be in contact with a high concentration of Hyal2, leading to activation of Env and refolding into a rod-like structure. Consequently, the low pH in these compartments results in further conformational change and six-helix bundle formation. This entry mechanism might be more favorable for an Env that folds into an unstable fusion intermediate such as JSRV Env.

Given the differential pH requirement for JSRV and ENTV Env, it is likely that they use different compartments for entry. As shown in chapter 3, ENTV infection is inhibited by the irreversible proton-pump inhibitor BafA1, yet is enhanced by a short treatment with reversible lysosomotropic agents, such as ammonium chloride. In addition, the use of the lysosomal protease inhibitor, leupeptin, also enhanced ENTV infection, yet had no effect on JSRV. This suggests that ENTV may fuse in the lysosomes and JSRV in an acidic vesicle upstream of the lysosomes, such as early or late endosomes. The use of live imaging and tracking of single viral particles would be useful in determining the exact entry pathway of JSRV and ENTV.
CONTRIBUTION TO GENERAL KNOWLEDGE

The work presented in this thesis revealed a multistep mechanism of fusion by oncogenic sheep retroviral envelopes that shares some similarities and important differences from that of ASLV. Furthermore, we uncovered new regulatory mechanisms that involve the cytoplasmic tail, the TM, and the SU subunits of Env. The major findings of these studies are listed below:

- We demonstrated for the first time that JSRV Env requires an acidic pH for entry and fusion activation. This is the first example of an oncogenic Env mediating fusion at low pH.

- We showed that JSRV Env fusion is modulated by inside-out signalling, whereby the N-terminal, but not the C-terminal portion of the cytoplasmic tail (CT) negatively regulates Env fusion activity. One of the possible mechanisms by which CT truncation enhances fusion activity is by modulation of the SU-TM interaction.

- We found that ENTV also requires a much lower pH for entry and fusion, and its fusion activity is lower than that of JSRV. This unusually low pH requirement likely explains the enhanced ENTV infection in the presence of lysosomal protease inhibitors. Among the enveloped viruses, ENTV represents the first example of a virus that requires such a low pH for activation, along with the recently characterized Lassa virus [23].

- We showed that the SU subunit of JSRV and ENTV Env modulates the fusion activity, and that the TM subunit dictates the pH requirement.
• We revealed that JSRV Env-mediated fusion requires the stepwise trigger of receptor binding and low pH. This is the second example of a pH-dependent viral fusion protein that needs the receptor for priming.

• We found that receptor binding leads to shedding of SU, a property different from ASLV, and renders the fusion protein sensitive to inhibition by a peptide analogous to the C-terminal heptad repeat. In addition, incubation with the receptor inactivates the viral infectivity. These findings suggest that Hyal2 alters the TM conformation by possibly adopting a rod-like structure. Overall, this regulatory mechanism used by JSRV for fusion might be important for viral oncogenesis.
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